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Award Number: W81XWH-05-1-0030

TITLE: Telomere Length as a Predictor of Aggressive Prostate Cancer

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CONTRACTING ORGANIZATION: Johns Hopkins University
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REPORT DATE: November 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Nov 2008	Final	1 Nov 2004 – 31 Oct 2008

4. TITLE AND SUBTITLE

Telomere Length as a Predictor of Aggressive Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0030

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
School of Medicine
Baltimore, MD 21205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We evaluated whether men with shorter telomere length in prostate cancer tissue and in normal appearing prostate tissue adjacent to adenocarcinoma have a higher risk of aggressive prostate cancer than men with longer telomere length. We included 663 men in the Health Professionals Follow-up Study who underwent radical prostatectomy and for whom tissue was obtained and arrayed on six tissue microarrays. We used FISH to conduct relative quantitation of telomere length in ~4000 digitally-imaged spots equating to ~40,000 records. For each man, we determined telomere length in normal stroma, normal epithelium (basal and luminal cells), cancer (luminal cells), high-grade prostatic intraepithelial neoplasia (basal and luminal cells), normal stroma associated with cancer, and normal stroma associated with PIN. The data were entered into software developed at Hopkins called Telometer. We are in the data cleaning phase, will perform the statistical analysis over the next few months, and will submit an abstract to a national cancer meeting next year while concurrently preparing the manuscript.

15. SUBJECT TERMS
Prostate cancer, telomeres, risk

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	19	**19b. TELEPHONE NUMBER** *(include area code)*
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INTRODUCTION

We are submitting this revised final report for our project entitled "Telomere length as a predictor of aggressive prostate cancer". We investigated whether telomere length in prostate cancer cells (prognostic factor) and in normal prostate epithelial cells (risk factor) predicts risk of aggressive prostate cancer, that is, prostate cancer with a greater potential to progress. We hypothesized that men with shorter telomeres are at higher risk of high-grade prostate cancer, biochemical failure, metastasis, and death from prostate cancer. We investigated whether telomere length in white blood cells is correlated with telomere length in the target tissue. We hypothesized a modest positive correlation. We are pleased to report that we have completed data collection and the statistical analysis and have submitted an abstract to a national meeting called The Role of Telomeres and Telomerase in Cancer Research, sponsored by the American Association for Cancer Research and to be held in February 27-March 2, 2010.

BODY

The aims of this proposal were to:

1. To test whether median telomere length in prostate cancer cells differs between men with aggressive prostate cancer and nonaggressive prostate cancer.

2. To test whether median telomere length in normal-appearing prostate epithelial cells differs between men with aggressive prostate cancer and nonaggressive prostate cancer.

3. To test whether median telomere length in normal-appearing prostate epithelial cells differs across levels of factors predicted to influence:
   a) Cellular proliferation – total energy intake, obesity, physical inactivity
   b) Oxidative stress – antioxidants (lycopene, selenium, vitamin E) and oxidants (cigarette smoking)

4. Secondary objective: To support the use of telomere length in peripheral blood lymphocytes as a surrogate for telomere length in prostate epithelium, we will determine the correlation between telomere length in peripheral blood lymphocytes and in normal-appearing prostate epithelial cells.

We had proposed that these aims be accomplished by the following tasks:

Task 1: Select study subjects from among participants in the Health Professionals Follow-up Study who have been diagnosed with prostate cancer, Months 1-4

Task 1 was completed. As we indicated previously, we made the decision to use tissue microarrays (TMAs) rather than individual sections per subject because of their greater efficiency. The total number of men included on the TMAs by the Harvard investigators was 663, which is greater than the 400 samples we had proposed to investigate. Efficiency in the use of TMAs came from the reduced number of slides (6 TMAs sections instead of 400 sections) that needed to be FISH stained for telomeres.

In September 2009, the HPFS investigators completed their biennial updating of their prostate cancer case (through January 31, 2006) and prostate cancer outcome (through March 30, 2009) files. This allowed us to use as the primary outcome men who died of their prostate cancers, a definitive outcome compared with the originally proposed outcome of aggressive prostate cancer (high Gleason sum, high clinical stage, or death from prostate cancer). The total number of cells for which telomere length was determined was 39,096. After exclusions (e.g., missing information on follow-up time and outcome), the final number of men whose samples contributed to the statistical analysis was 623. Of these 48 died of their prostate cancer over a median of 10 years since surgical treatment for their prostate cancer.

Task 2: Obtain biological samples for study subjects, Months 2-6

The tissue microarray sections were received in September 2006. The delay was due to the time it took for Harvard to construct the TMAs, which was done for a variety of projects in addition to ours. We also made a change in what we requested for the white blood cells: rather than request blood specimens from which we had planned to extract DNA for Aims 3 and 4, we took advantage of concurrent work ongoing in the Health Professionals Follow-up Study (HPFS) and requested already extracted DNA from not just the men whose tissues had been includes on the TMAs but on all men diagnosed with prostate cancer during the same time frame as the cases as well as on matched controls (which includes the men on the TMAs). This request allowed us to address a new aim in additional to Aims 3 and 4: To test whether median telomere length in peripheral white blood differs between men with and without prostate cancer. This request involved coordinating the return of the HPFS DNA specimens from the National Cancer Institute's Cohort Consortium genotyping efforts.

The DNA extracted from white blood cells was retrieved from NCI and was delivered to the laboratory of Dr. Immaculata DeVivo at Harvard, where telomere length was determined.

Task 3: Determination of telomere length in prostate cancer cells (Aim 1) and in normal adjacent epithelial cells (Aim 2), Months 7-22

We first documented our ability to stain (FISH) for telomeres in normal epithelium and in foci of adenocarcinoma using the HPFS TMAs. Then, we imaged approximately 4,000 spots on a total of 6 TMAs. The imaging process was extraordinarily labor intensive, in part, because the contrast of the staining colors for telomeres, centromeres, high molecular weight cytokeratins (which are indicative of the basal layer of the epithelium), and DAPI (fraction of the area that is nuclear) needed to be optimized manually for each image. To increase the rate at which we can image the spots and assess telomere length, we hired an additional investigator, a genitourinary pathology fellow (Dr. Chilsuk Yoon). We also purchased a new tool for this work, a touch screen Thinkpad computer, on which the fellow could circle the array of the image (normal or tumor) for which

telomere length would be quantitated. This improved the speed the work. After Dr. Yoon's departure, we included another pathology fellow in the work (Dr. Thomas Lee).

For each of the 663 men, we determined telomere length in normal stroma, normal epithelium (basal and luminal cells), cancer (luminal cells), high-grade prostatic intraepithelial neoplasia (basal and luminal cells), normal stroma associated with cancer, and normal stroma associated with PIN. We entered the data were entered into software developed at Hopkins called Telometer: Software for Telomere Counting (http://bui2.win.ad.jhu.edu/telometer/). This software extracts telomere length from the fluorescence images using the analysis program ImageJ (NIH software). The software was conceptualized by co-investigators Drs. Meeker and De Marzo, was programmed by Joe Zimmerman, and is now maintained by James Morgan.

Task 4: Determination of telomere length in lymphocytes, Months 23-30

We determined that direct *in situ* labeling of spotted cells was not adequately reliable for the proposed work. We subsequently identified two alternative methods for this aim, one from the published literature (Cawthon RM. Nucleic Acids Research 2002; 30:e47. PMID 12000852) and one that we developed that utilizes padlock probe technology. We conducted side-by-side comparison for validity and reliability. Dr. Platz provided Dr. Meeker with samples from her biorepository for this work. During the time frame of this project, major improvements in high-throughput technologies for assessment of telomere length in extracted DNA occurred elsewhere. Thus, we changed the laboratory from Dr. Meeker (an expert in fluorescent in situ hybridization (FISH)-based determination of telomere length in tissue) to Dr. Immaculata De Vivo at Harvard University. She uses a 384-well plate, real-time PCR on an Applied Biosystems 7900 HT Thermocycler to estimate relative average telomere length, which is based on the work by Cawthon above. Details of her approach have been published previously (Han J et al. J Investigative Dermatology 2008; epub ahead of print. PMID: 18668136). We were pleased with this new collaboration and Dr. De Vivo has agreed to help us implement her approach for future telomere length studies that we may conduct using extracted DNA at Hopkins.

The De Vivo laboratory completed the white blood cell telomere length determinations and delivered a dataset to Dr. Platz for statistical analysis.

Task 5: Data management and interim analysis, Months 25-30

We directly exported the tissue telomere length data from Telometer into an MS Access database. From there, we exported the data into Excel for data analysis. The final number of records was 39,096. Extensive data management was required to produce a dataset that was adequately cleaned for statistical analysis. We were fortunate that the Prostate Cancer SPORE TMA Core Facility programmer (James Morgan) who co-developed TMAJ (http://tmaj/pathology.jhmi.edu) with co-investigator Dr. De Marzo and who currently manages Telometer was helped us to manipulate this complex dataset.

Dr. Platz began writing the program to perform the statistical analysis in late 2008. Additional data cleaning was required. Extensive analysis was performed for the original endpoint of aggressive disease. Monthly meetings were held to discuss the approach, relevant cell comparisons, and to review interim results. Major revisions to the analysis were done in July 2009, when an early version of the biennially updated HPFS case file became available. In November 2009, the program was again substantially revised to take full advantage of the data on case-fatality, which now was of sufficient size for a stable analysis. The team met in person weekly along with email and telephone calls to guide the analysis and inference phase.

On 12/15/2009 we submitted an abstract entitled "Greater variability in telomeres in cancer cells and shorter telomeres in cancer-associated stromal cells are associated with a higher risk of prostate cancer death in surgically-treated men" describing the results of this study to a national meeting.

An Excel spreadsheet was received from the De Vivo laboratory providing the results for white blood cell telomere length. Dr. Platz these data were merged with the HPFS prostate cancer case file and with the tissue telomere length data. She determined the correlation between tissue and white blood cell telomere length. She also evaluated the association between white blood cell telomere length and prostate cancer risk, advanced prostate cancer risk, pathologically localized disease, Gleason 7+ disease, Gleason < 7 disease, and risk of recurrence. She also evaluated whether white blood cell telomere length was associated with advanced prostate cancer compared with pathologically localized disease.

Task 6: Final analysis and report/manuscript preparation, Months 31-36

Although the work took substantially longer to complete than planned, the stumbling points and delays, in the end, led to much more informative results that have substantial translational potential. Detailed results are found under **REPORTABLE OUTCOMES**.

KEY RESEARCH ACCOMPLISHMENTS

•   Completed the work for Aims 1, 2, 4, and a new aim (Is shorter white blood cell telomere length associated with risk of prostate cancer, advanced prostate cancer, and prostate cancer recurrence). Submitted an abstract to a national meeting. The work for aim 3 is pending. As we prepare the manuscripts(s) for Aims 1, 2, 4 and the new aim, we will continue the analyses to fully characterize factors associated with tissue and white blood cell telomere length.

•   Other accomplishments of the research team, which were enhanced by the collaboration funded by DOD. During the course of this project, we began a joint laboratory meeting that meets once per month. Participants are the investigators on this

DOD-funded project (Drs. Platz (epidemiology), De Marzo (pathology), Meeker (cancer biology)) as well as other prostate cancer investigators (Drs. Drake (immunology) and Yegnasubramanian (cancer biology)), doctoral students, and fellows. We review the status of each project, brain storm about solutions to problems, and develop new research questions, some of which have culminated in grant application submissions.

o   The Hopkins research team continues to collaborate effectively on the etiology of prostate cancer, other prostate diseases, and other cancers.

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o   Dr. Meeker has continued to publish on telomeres, telomerase, and related pathways in cancer.

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o   Drs. Platz (Hopkins) and Giovannucci (Harvard) continue to collaborate on prostate cancer, other prostate diseases, and other cancers in the HPFS (exclusive of those already listed above). Many of these factors are hypothesized to influence either cell proliferation or oxidation and thus could affect telomere length. In the future we will investigate whether these exposures or biomarkers are correlated with telomere length either in tissue or peripheral blood lymphocyte DNA.

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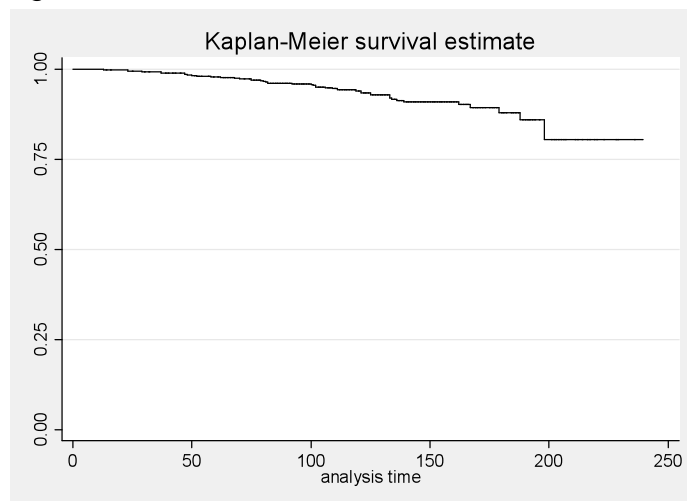
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REPORTABLE OUTCOMES

Aims 1 and 2: The final sample size was 623 surgically-treated men in the HPFS of whom 48 died of their prostate cancers. The median follow-up was 10 years. Shown in Figure 1 is the overall prostate cancer-specific survival in the 623 men with prostate cancer in the HPFS (analysis time is in months since prostate cancer diagnosis), irrespective of telomere length.
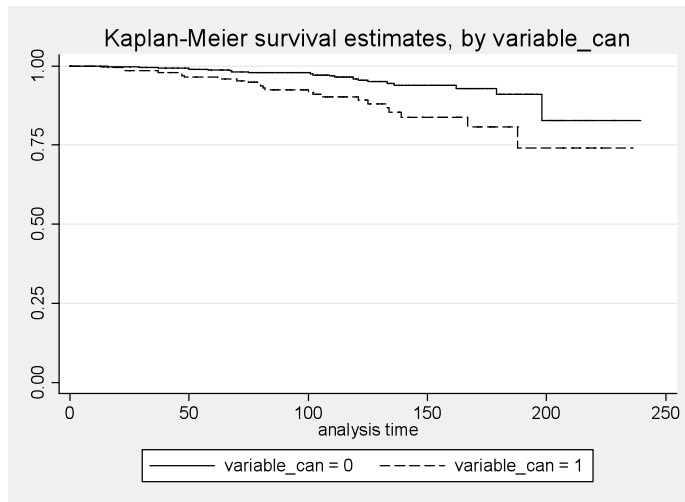
Figure 1.

Shown in Figure 2 is the primary result of Aim 1: Men whose prostate cancer cells had
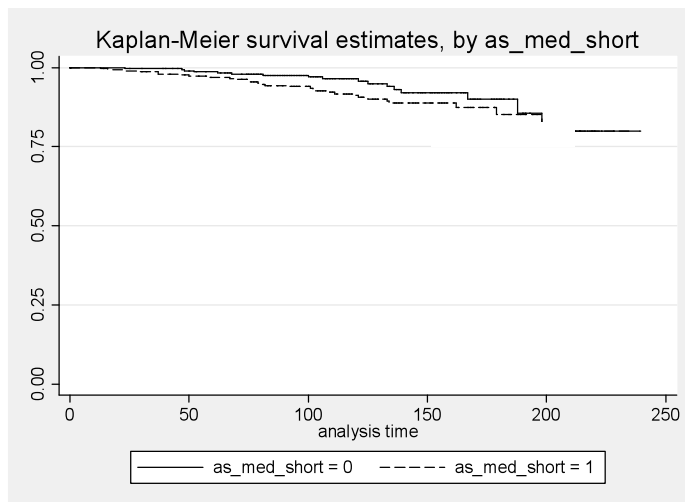
greater variability in telomere length (top third – dashed line) were more likely to die of their prostate cancer than men whose prostate cancer cells had less variability in telomere length (middle and bottom thirds – solid line). This difference was statistically significant (log-rank p=0.002).

Figure 2.

Shown in Figure 3 is the primary result of Aim 2: Men whose cancer-associated stromal cells had shorter telomeres (bottom and middle thirds – dashed line) were slightly more likely to die of their prostate cancer than men whose cancer-associated stromal cells had longer telomeres (top third – solid line). This difference was not statistically significant (log-rank p=0.09).
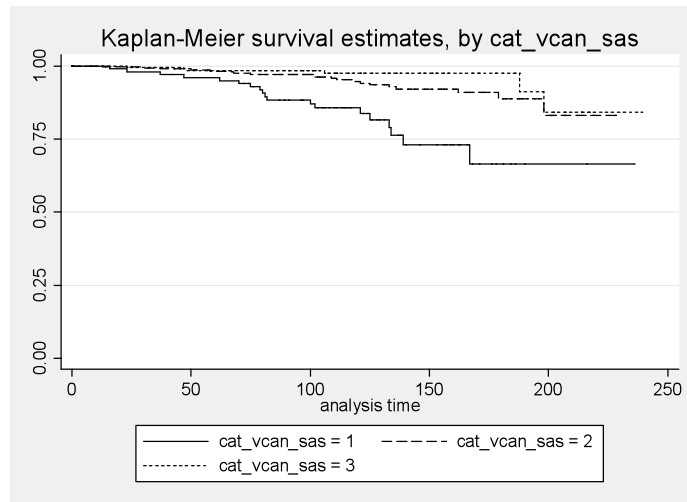
Figure 3.

We then combined the results from Aims 1 and 2 to generate the results shown in Figure 4: Men whose prostate cancer cells had greater variability in telomere length and

whose cancer-associated stromal cells had shorter telomeres (solid line) were more likely to die of their prostate cancer than men whose prostate cancer cells had less variability in telomere length and/or whose cancer-associated stromal cells had longer telomeres (dotted lines). This difference was statistically significant (log-rank p<0.0001).

Figure 4.

We then estimated the hazard ratio (RR) of prostate cancer death and 95% confidence intervals for variable telomere length in cancer cells and short telomeres in cancer-associated stromal cells using Cox proportional hazards regression adjusting for age and year of diagnosis, pathological stage, and Gleason sum (Table 1). There was no association between the length of the telomeres in cancer cells and death from prostate cancer. However, men who had more variable length telomeres in their cancer cells were more than 2 times more likely to die of their prostate cancer. Men whose cancer associated stromal cells had shorter telomeres were 4 times more likely to die of their prostate cancer.

In a post-hoc analysis, we combined over tertiles that had similar RRs and entered into the model terms for both variable telomeres in cancer and short telomere in cancer-associated stromal cells (Table 1). After mutual adjustment, men with variable telomeres in cancer had 3 times the risk and men with short telomeres in cancer-associated stromal cells had 6 times the risk of prostate cancer death.

Measurement	Tertile of telomere length			p-trend	Mutual adjustment**
Median of cancer cells					
No. cases / PY	15/26,543	10/24,331	22/22,539		
RR	0.71	0.46	1.00	0.18	NA
95% CI	0.36-1.38	0.21-1.00	Reference		
Standard deviation of cancer cells					T3 vs T1/T2
No. cases / PY	11/26,107	11/24,018	24/23,117		24/23,117 vs 22/50,125
RR	1.00	1.16	2.61	0.004	3.24
95% CI	Reference	0.50-2.68	1.26-5.39		1.76-5.97
Median of cancer-associated stromal cells					T1/T2 vs T3
No. cases / PY	18/26,207	23/23,564	7/24,029		40/49,376 vs 6/23,866
RR	4.12	4.58	1.00	0.003	6.44
95% CI	1.61-10.51	1.87-11.24	Reference		2.57-16.13

Table 1. Association* between telomere length in prostate tissue and risk of death from prostate cancer after prostatectomy, Health Professionals Follow-up Study

*Adjusted for age (continuous) and year (ordinal) at diagnosis, pathologic Gleason sum (ordinal), and stage (advanced vs nonadvanced)
**Among men who had both variability in cancer cell telomere length and median cancer-associated stromal cell telomere length and entered into the model simultaneously; when not mutually adjusted the associations were 2.43 (1.34-4.40) and 5.15 (2.05-12.92), respectively.

We next combined variability in cancer cell telomere length with telomere length in cancer-associated stromal cells (Table 2). Using the cutpoints from Table 1 under the column "mutual adjustment", we generated Combination 1. Only 1 man who did not have variable telomeres in cancer and did not have short telomeres in cancer-associated stromal cells did not die of his prostate cancer, thus this analysis did not yield stable estimates. Nevertheless, it can be seen that men who had both variable cancer telomeres and short cancer-associated stromal cell telomeres had a substantially higher risk of dying of their prostate cancer and importantly, men who did not have variable cancer telomeres and did not have short cancer-associated stromal cell telomere were very unlikely to die of their prostate cancers. We next tried several other combinations to increase the stability of the findings. The findings of these analyses were all consistent: men with more variable telomere lengths in cancer cells and shorter telomere lengths in cancer-associated stromal cells are more likely to die of their prostate cancers. Combination 3 is the same as shown in Figure 4.

Table 2. Association* between combinations of variability in cancer cell telomere length and telomere length in cancer-associated stromal cells in prostate tissue and risk of death from prostate cancer after prostatectomy, Health Professionals Follow-up Study

	Cancer cells / cancer-associated stromal cell telomeres			
Combination 1**	Not variable / not short	Not variable / short	Variable / not short	Variable / Short
No cases/PY	1/12,306	21/37,819	5/11,560	19/11,557
RR	1.00	14.14	8.37	41.09
95% CI	Reference	1.77-113.2	0.94-74.71	5.10-331.2
Combination 2**	All others	-	-	Variable / Short
No cases/PY	27/61,685	-	-	19/11,557
RR	1.00	-	-	4.31
95% CI	Reference	-	-	2.33-8.00
Combination 3**	Not variable / not short or variable / not short	Not variable / short	-	Variable / Short
No cases/PY	6/23,866	21/37,819	-	19/11,557
RR	1.00	3.48	-	10.14
95% CI	Reference	1.32-9.19	-	3.78-27.22
Combination 4***	Not variable / not short	Not variable / short	Variable / not short	Variable / Short
No cases/PY	4/26,863	4/34,648	26/8695	12/3,036
RR	1.00	2.45	2.02	10.52
95% CI	Reference	1.18-5.09	0.61-6.61	3.04-36.37
Combination 5***	All others	-	-	Variable / Short
No cases/PY	34/70,206	-	-	12/3.036
RR	1.00	-	-	5.59
95% CI	Reference	-	-	1.89-16.56

*Among men who had measurements of variability in cancer cell telomere length and cancer-associated stromal cell telomere length and adjusted for age (continuous) and year (ordinal) at diagnosis, pathologic Gleason sum (ordinal), and stage (advanced vs nonadvanced)

**Variable cancer cell telomere length in top tertile; not variable cancer cell telomere length in bottom and middle tertiles; short telomere length in cancer-associated stromal cells in bottom and middle tertiles; not short telomere length in cancer-associated stromal cells in top tertile

***Variable cancer cell telomere length in top sextile; not variable cancer cell telomere length in bottom 5 sextiles; short telomere length in cancer-associated stromal cells below median; not short telomere length in cancer-associated stromal cells at or above median

Aim 3 is pending.

For Aim 4, we found that white blood cell telomere length was positively correlated with variability in telomere length in prostate cancer cells (Spearman r=0.70, p=0.0039) and was inversely correlated with telomere length in normal stromal cells (r=-0.49, p=0.07), normal basal cells (r=-0.56, p=0.03), and cancer-associated stromal cells (r=-0.53, p=0.04). White blood cell telomere length was not correlated with prostate cancer cell telomere length normal luminal cells or cancer associated luminal cells.

For the new aim, first we documented that age was inversely associated with white blood cell telomere length in the controls (Spearman r=-0.15, p=0.07). We then compared white blood cell telomere length between the prostate cancer cases to controls and between the advanced cases and controls. Telomere length was statistically significantly shorted in the advanced cases than in the controls, but there was no difference between total prostate cancer cases and the controls (Table 3).

Table 3. Telomere Length in Prostate Cancer Cases and Control Nested in the Health Professional Follow-up Study

	Controls	Cases		P-value (versus controls)	
		Total	Advanced	Total	Advanced
Number	135	158	18		
Mean ± sd age at blood draw (yr)	66.4 ± 7.1	65.8 ± 7.1	70.1 ± 5.8	0.39	0.09
Telomere length*					
Mean ± sd	6.5 ± 1.8	6.7 ± 2.4	5.3 ± 1.8	0.56**	0.02**
Median (IQR)	6.4 (5.1-7.6)	6.1 (5.1-7.7)	5.4 (3.7-6.2)	0.94	0.009

* Interpreted as the number of times longer the telomere amplicon was than the single copy gene amplicon.
**Adjusted for age at blood draw these p-values were 0.64 and 0.02, respectively

Next, we evaluated the association between white blood cell telomere length and risk of prostate cancer. We found that white blood cell telomere length was not associated with risk of prostate cancer overall, pathologically organ-confined disease, or risk of high or low Gleason sum, but men who had short telomeres were more likely to be diagnosed with prostate cancer that was advanced stage at diagnosed, to progress, or to cause death (Table 4). The association appeared to be the strongest when comparing advanced cases to those that were localized. White blood cell telomere length was not associated with risk of prostate cancer death after prostatectomy (data not shown).

Table 4. Association between white blood cell telomere length and risk of prostate cancer, Health Professionals Follow-up Study

Prostate cancer	Tertile of white blood cell telomere length			p-trend
Total vs controls	52/45	53/46	53/44	
OR*	0.99	0.99	1.00	0.98
95% CI	0.56-1.76	0.56-1.74	Reference	
Advanced** vs controls	10/45	5/46	3/44	
OR*	2.80	1.39	1.00	0.11
95% CI	0.73-11.4	0.31-6.24	Reference	
Advanced** vs localized	10/42	5/48	3/50	
OR*	3.72	1.61	1.00	0.05
95% CI	0.94-14.7	0.36-7.23	Reference	

*Adjusted for age at blood draw and for history of PSA screening before blood draw
**Advanced at diagnosed or during follow-up or fatal

CONCLUSIONS

- Greater variability in telomere length in prostate cancer cells and short telomere length in cancer-associated stromal cells was associated with a higher risk of prostate cancer death in men surgically treated for prostate cancer. These findings were independent of pathologic prognostic indicators. We anticipate that determination of telomere length at the time of prostate cancer diagnosis and/or surgery coupled with information on stage and grade will enhance the ability to predict prognosis. If so, then it may be possible to make better decisions about who to treat and the intensity of treatment beyond the current approach.

- White blood cell telomere length was inversely correlated with normal and cancer-associated stromal cell telomere length and positively correlated with the variability in telomere length in cancer cells. However, white blood cell telomere length was not associated with risk of prostate cancer death after prostatectomy. This result suggests that measurement of white blood cell telomere length may not have clinical utility in predicting risk of prostate cancer with the worst phenotype.

REFERENCES

- None

APPENDICES

- None