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TITLE: Realizing the Translational Potential of Telomere Length Variation as a Tissue-Based Prognostic Marker for Prostate Cancer

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14. ABSTRACT We are testing, in prospective studies from Hopkins (Brady) and Harvard (PHS, HPFS), whether the combination of telomere length variability in prostate cancer cells and short telomere length in cancer-associated stromal cells is an independent prognostic indicator of poor prostate cancer outcome. We will automate the method for measuring telomere length and find optimal prognostic cutpoints. In Year 1, Hopkins purchased a state-of-the art fluorescence slide scanner and associated image analysis software using donor funds. We will use this scanner/software in place of older technology at Harvard. Given the technology change, we held 2 in-person meetings to map out a new strategy for the work. We felt it was prudent to delay starting the project while installing the scanner and training the team to use the scanner/software for telomere length determination in individual cells. We requested sections from an HPFS test TMA for optimizing the automated method and comparing the automated and manual methods. The 16 Brady TMAs were sectioned/mounted for FISH staining. We requested sections from the 6 PHS and 3 new HPFS TMAs. While delayed, the newer technology will help us best achieve the aims of this project.					
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INTRODUCTION: Currently used clinico-pathologic prognostic factors are imperfect predictors of outcome in the men with clinically localized prostate cancer, the majority of men diagnosed today. Thus, tissue-based biomarkers that significantly enhance the predictive power are urgently needed to improve treatment and surveillance decision-making for these men. To address this pressing clinical need, we have assembled a multidisciplinary prostate cancer research team from Johns Hopkins and Harvard to validate and optimize a novel tissue biomarker of prognosis for men with clinically localized prostate cancer that we recently identified – telomere length variability in prostate cancer cells combined with short telomere length in cancer-associated stromal cells. In our prior work, men with this combination had a substantially higher risk of dying of their prostate cancer compared with men without this combination. Equally importantly, men without this combination rarely died of their prostate cancer over 10 years. Key next steps to realize the great translational potential of telomere length as an independent prognostic tissue biomarker are optimized biomarker assessment and validation. Thus, our aims are to: 1) Optimize the method for assessing telomere length by FISH using a high-throughput approach to yield a test feasible for the clinical setting. 2) Validate our compelling findings in two other cohort studies on prostate cancer outcomes: a) men surgically treated and followed for lethal prostate cancer; and b) men surgically treated and followed for prostate cancer recurrence. 3) Determine optimal biomarker cutpoints for prognosis.

BODY: This work is being performed collaboratively by two institutions: Johns Hopkins Bloomberg School of Public Health and Harvard School of Public Health. We obtained all required IRB approvals for both the Brady prostate cancer recurrence Study (Hopkins), the Physician's Health Study (PHS) and the Health Professionals Follow-up Study (HPFS, Harvard), including from the DOD IRB (**Task 1 completed**). Drs. Platz and De Marzo previously created the Brady prostate cancer recurrence nested case-control study (Brady study; in part with prior DOD funding to Dr. Platz at Hopkins) and associated tissue microarrays (TMAs). This TMA set is now part of the Prostate Cancer Biorepository Network (PCBN). For equitable use and tracking purposes, during Year 1, we applied for access to these TMAs and received approval from the PCBN. For the Brady study, we pulled the recurrence TMAs (N=16 TMAs, which includes 524 cases and 524 controls) and cut and mounted the sections (**Task 4b completed**) for staining for telomere-specific FISH, cytokeratin 903 immunofluorescence, and for DAPI. The PHS and HPFS are existing cohort studies, and TMAs have been constructed for those participants who underwent a radical prostatectomy with other funds, including previous DOD funding (to Dr. Mucci at Harvard). We requested sections from the 6 PHS TMAs and 3 HPFS TMAs that had not yet been constructed when we conducted our prior work on the telomere biomarker and that served as the preliminary data for the proposal for the current study.

In Year 1, the Johns Hopkins investigators purchased a new state-of-the art fluorescence slide scanner and associated image analysis software from Tissue Gnostics using donor funds. This new system uses the TissueFAXS Plus (Tissue Gnostics, Vienna, Austria) microscopy workstation for slide-based cytometry of tissue sections and TMAs. The microscope is a Zeiss Z2 Axioimager with high quality optics applicable for fluorescence imaging. The microscope is fitted with the following filter sets: DAPI, Alexa 488/Cy2, Alexa 568/Cy3, and Alexa 633/Cy5. The system contains an ultra-precise motorized stage for 8 slides for high-throughput scanning. In addition, a separate image analysis workstation contains a high-performance computer workstation (HP Z420 configured with 6 cores) and includes the TissueFAXS 4.0, TissueQuest 4.0, and HistoQuest 4.0 software modules (Tissue Gnostics).

Previously, Johns Hopkins did not have such a scanner and thus, we had originally proposed to use a fluorescence slide scanner that was available at Harvard. The Hopkins and Harvard investigators agreed that the new scanner represents superior technology and should be used in place of the older technology scanner at Harvard, which we would have had to modify, both the scanner and the software, for the measurement of telomere length in individual cells. We selected the Tissue Gnostics scanner and image analysis software because they do not require such modification. Given the change in the technology to be used, 2 in-person meetings were held between the Hopkins and Harvard investigators to determine next steps. We felt it was prudent to delay the start of the work on this project while installing the scanner and getting the team up to speed on use of the scanner and image analysis software for telomere length determination in individual cells.

During the 2 in person meetings between the Hopkins and Harvard investigators, we determined it was appropriate to add:

- 1) Addition to Task 2: Optimize method for telomere length determination in individual cells in tissue microarrays constructed from formalin-fixed paraffin embedded tissue using FISH. The HPFS men are from across the US, which means that the processing of their prostatectomy samples is not standardized (unlike the test TMAs that we are using from our Johns Hopkins patients). To have clinical implementation, it is critical to optimize our method of telomere length determination using tissue processed using different protocols.
- 2) Addition to Task 3: Test the automated method of telomere length determination for precision and validity by re-running the automated method (precision) and by comparing to the nonautomated method (validity). The original 5 HPFS TMAs that we stained using a fluorescent telomere-specific oligonucleotide fish probe have been stored refrigerated for several years. It is important for future testing of the telomere biomarker (e.g., testing of new platforms using the same already stained TMA sections) to know whether storage time affects the intensity of the FISH signal. Thus, we will compare the signal intensities between the original and new scans of the same HPFS TMAs.
- 3) Addition to Task 3: Test the automated method of telomere length determination for precision and validity by re-running the automated method (precision) and by comparing to the nonautomated method (validity). It is important to know whether sequential cuts through the same tissue block would yield the same risk classification of the telomere biomarker. Thus, we have requested new cuts of the 5 HPFS TMAs to be able to compare between the original TMA section and the new TMA section from the same paraffin block to determine agreement in telomere biomarker classification.
- 4) Addition to Tasks 4, 5, 6: 3 additional HPFS TMAs have been constructed since we performed our original study. We have requested sections of these TMAs and will include these TMAs in the current work.

The modified Statement of Work is attached with all of the changes described above shown.

KEY RESEARCH ACCOMPLISHMENTS: None directly from this project to date (see above).

Subsequent to the submission of this DOD grant, our preliminary study was published in *Cancer Discovery*: Heaphy CM, Yoon GS, Peskoe SB, Joshu CE, Lee TK, Giovannucci E, Mucci LA, Kenfield SA, Stampfer MJ, Hicks JL, De Marzo AM, Platz EA, Meeker AK. Prostate cancer cell telomere length variability and stromal cell telomere length as prognostic markers for metastasis and death. *Cancer Discov* 2013;3:1130-41. PMID: 23779129. This study was primarily funded by a prior DOD grant (W81XWH-05-1-0030).

In addition, our team studied telomere length in circulating leukocytes as a possible biomarker for the risk of prostate cancer: Hurwitz LM, Heaphy CM, Joshu CE, Isaacs WB, Konishi Y, De Marzo AM, Isaacs SD, Wiley KE, Platz EA, Meeker AK. Telomere length as a risk factor for hereditary prostate cancer. *Prostate* 2013 Nov 28. doi: 10.1002/pros.22755. [Epub ahead of print] PMID: 24285042. This study was primarily funded by a prior DOD grant (W81XWH-06-1-0052).

REPORTABLE OUTCOMES: None

CONCLUSION: None to date, as consistent with the Statement of Work.

REFERENCES: None

APPENDICES: None

SUPPORTING DATA: None

REVISED Statement of Work

CHANGES TO THE STATEMENT OF WORK ARE SHOWN IN UPPER CASE FONT AND ~~STRIKETHROUGH~~.

DURING YEAR 1, HOPKINS PURCHASED A NEW STATE-OF-THE-ART PIECE OF FLUORESCENCE SLIDE SCANNER AND ASSOCIATED IMAGE ANALYSIS SOFTWARE USING DONOR FUNDS (RAISED BY PLATZ AND MEEKER) THAT NECESSITATES CHANGES TO THE STATEMENT OF WORK. BY AGREEMENT WITH THE HARVARD SUBCONTRACT PI (MUCCI), THIS NEW SCANNER/SOFTWARE WILL BE USED AT HOPKINS IN PLACE OF THE PREVIOUSLY PLANNED USE OF OLDER TECHNOLOGY AT HARVARD.

WE ALSO MODIFIED THE FREQUENCY OF ATTENDENCE AT THE IMPACT MEETING TO ONE TIME, PER THE NEGOTIATION AT THE TIME OF THE AWARD.

Work will be performed at two institutions:

- 1) Johns Hopkins Bloomberg School of Public Health
Department of Epidemiology
615 N. Wolfe St.
Baltimore, MD 21205
- 2) Harvard School of Public Health
Department of Epidemiology
667 Huntington Ave.
Boston, MD 02115

In the list of tasks below, the location of the work to be performed is indicated in parentheses.

Task 1.

Aims 1-4: Prepare and submit IRB protocol amendments; months 1-4.

- a. Prepare and submit amendment for Aims 2 and 3 to Hopkins IRB protocol H.34.02.11.08.A2 [Brady Prostate Cancer Recurrence Nested Case-Control Study]. (Hopkins)
- b. Prepare and submit amendment for Aims 2 and 3 to Harvard IRB protocol 2009-P-001231/5 [Physicians' Health Studies and Health Professionals Follow-up Study joint protocol for molecular studies]. (Harvard)
- c. Prepare and submit amendment for Aim 3 to Hopkins IRB protocol H.34.04.07.28.A2 [Health Professionals Follow-up Study telomeres protocol]. (Hopkins)
- d. Prepare and submit application to DOD IRB. (Hopkins)

Task 2.

Aim 1: Optimize method for telomere length determination in individual cells in tissue microarrays constructed from formalin-fixed paraffin embedded tissue using FISH; months 5-12.

- a. CUT 2 SECTIONS OF THE HPFS TEST TMAS FOR USE IN OPTIMIZATION AND AUTOMATION. SHIP TO HOPKINS. (HARVARD)
- b. STAIN THESE TMA SECTIONS USING A FLUORESCENT TELOMERE-SPECIFIC OLIGONUCLEOTIDE FISH PROBE, IMMUNOFLUORESCENCE FOR BASAL CELL-SPECIFIC CYTOKERATINS, AND STAIN FOR TOTAL NUCLEAR DNA WITH DAPI. (HOPKINS)
- c. PURCHASED A FLUORESCENT SLIDE SCANNER AND IMAGE ANALYSIS SOFTWARE FROM TISSUE GNOTICS WITH DONOR FUNDS. THIS EQUIPMENT ELIMINATES THE NEED FOR THE COLLABORATIONS WITH Cri Vectra STEP. OPTIMIZE THE PERFORMANCE OF NEW THE FLUORESCENT SLIDE SCANNER USING BOTH HOPKINS TEST TMAS AND HPFS TEST TMAS. (HOPKINS) ~~Collaborate with Cri Vectra to modify the automated fluorescence slide scanner; upgrade to 40x magnification. (Harvard and Hopkins)~~
- d. Use THE TISSUE GNOTICS ~~Defines~~ image analysis software to automate segmentation of individual cell nuclei and telomere FISH signals, AS WELL AS BASAL EPITHELIAL CELLS BASED ON CYTOKERATIN ANTIBODY STAINING. (Harvard and Hopkins)

Task 3.

Aim 1: Test the automated method of telomere length determination for precision and validity by re-running the automated method (precision) and by comparing to the nonautomated method (validity); months 13-18.

- a. CUT AND SEND 1 NEW SECTION OF EACH OF THE 5 HEALTH PROFESSIONALS FOLLOW-UP STUDY TMAS THAT WE USED IN OUR PRIOR STUDY. (HARVARD)
- b. STAIN THE 5 NEWLY CUT TMA SECTIONS USING A FLUORESCENT TELOMERE-SPECIFIC OLIGONUCLEOTIDE FISH PROBE AND STAIN FOR TOTAL NUCLEAR DNA WITH DAPI. (HOPKINS)
- c. Scan the 5 ORIGINAL HPFS TMAS AND 5 NEW CUTS OF THE HPFS TMAS using THE TISSUE GNOTICS FLUORESCENCE slide scanner. (Harvard HOPKINS)
- d. ~~Transfer the scanned digital images to Hopkins. (Harvard)~~
- e. ~~Upload the digital images and~~ Perform image analysis using the TISSUE GNOTICS IMAGE ANALYSIS software, INCLUDING DETERMINING IF STORAGE TIME OF THE STAINED SECTIONS AFFECTS ABILITY TO DETERMINE TELOMERE LENGTH (E.G., LOSS OF STAINING INTENSITY BETWEEN THE ORIGINAL SCAN AND THE CURRENT SCAN OF THE SAME HPFS TMA SECTIONS). (Hopkins)
- f. Repeat c (scanning), d, and e; calculate the mean of the men's coefficients of variation for telomere length in cancer cells and cancer associated stromal cells across the two runs to assess precision. (Hopkins)
- g. Perform man by man comparisons of telomere length as assessed using the automated and the nonautomated methods using paired statistics and the Kappa statistic. Use the data previously obtained through the nonautomated method as the gold standard. (Hopkins)
- h. IF STORAGE TIME DID NOT AFFECT STAINING INTENSITY, PERFORM MAN BY MAN COMPARISONS OF TELOMERE LENGTH USING THE AUTOMATED METHOD AS ASSESSED IN THE OLD VERSUS NEW TMA SECTIONS (SAME BLOCKS BUT DIFFERENT CUT THROUGH THE BLOCKS) USING THE SAME METHODS AS g. ABOVE. (HOPKINS)
- i. Revisit Task 2, as needed, to continue to optimize the method for precision and validity.

Task 4.

Aim 2: Prepare the Physicians' Health Study (PHS) Lethal Prostate Cancer Cohort Study (6 TMAs), THE HPFS (3 NEW TMAS), and the Brady Prostate Cancer Recurrence Nested Case-Control Study TMAs (16 TMAs) for determination of telomere length; months 9-18.

- a. Pull the existing PHS Lethal Prostate Cancer Cohort Study TMAs. Cut 1 section of each of the 6 TMAs. CUT 1 SECTION OF EACH OF THE 3 NEWLY MADE HPFS TMAS. Ship to Hopkins. (Harvard)
- b. Pull the Brady Prostate Cancer Recurrence Nested Case-Control Study TMAs from the TMA facility. Cut 1 section of each of the 16 TMAs. (Hopkins)
- c. Stain the 25 TMA sections using a fluorescent telomere-specific oligonucleotide FISH probe and stain for total nuclear DNA with DAPI. ~~Send the stained TMAs to Harvard.~~ (Hopkins)
- d. Prepare annual DOD progress report. (Hopkins)
- e. Prepare annual IRB progress reports (3 protocols). (Hopkins and Harvard)
- f. ~~Attend IMPaCT meeting. (Hopkins and Harvard)~~

Task 5.

Aim 2: Determine telomere length for the PHS Lethal Prostate Cancer Cohort (45 lethal cases in 500 men), THE TWO NEWLY MADE HPFS TMAS, and the Brady Prostate Cancer Recurrence Nested Case-Control Study (524 recurrence cases and 524 controls) using the TISSUE GNOSTICS FLUORESCENCE SLIDE SCANNER ~~modified slide scanner~~ and image analysis software; months 19-36.

- a. Scan the 22 FISH-stained TMAs using the TISSUE GNOSTICS FLUORESCENCE SLIDE SCANNER. (Harvard)
- b. ~~Upload the digital images into the modified image analysis software. (Hopkins)~~
- c. Perform image analysis for the PHS Lethal Prostate Cancer Cohort TMAs AND THE 3 NEWLY MADE HPFS TMAS. SEND THE DATA TO HARVARD. ~~(Harvard Hopkins)~~
- d. Perform image analysis for the Brady Prostate Cancer Recurrence Nested Case-Control Study TMAs. (Hopkins)
- e. Prepare annual DOD progress reports for month 24 and month 36. (Hopkins)
- f. Prepare annual IRB progress reports (3 protocols) on two occasions. (Hopkins and Harvard)
- g. ~~Attend IMPaCT meeting. (Hopkins and Harvard)~~

Task 6.

Aim 2: Perform statistical analysis of the association between telomere length and risk of lethal prostate cancer and prostate cancer recurrence and prepare manuscripts. Perform in parallel between the two cohorts; months 37-42.

- a. Perform data management. Calculate reliability measures for subset of re-reviewed samples. (Hopkins and Harvard)
- b. Perform statistical analyses using Cox proportional hazards regression modeling for the PHS Lethal Prostate Cancer Cohort Study. (Harvard)
- c. Perform the statistical analyses using conditional logistic regression for the Brady Prostate Cancer Recurrence Nested Case-Control Study. ADD THE NEW HPFS DATA TO THE ORIGINAL HPFS DATA THAT FORMED THE BASIS FOR THIS WORK. (Hopkins)

- d. Present findings at national cancer meetings ~~and IMPaCT meeting~~. (Hopkins and Harvard)
- e. Write manuscripts, one for lethal prostate cancer and one for prostate cancer recurrence. (Hopkins and Harvard)

Task 7.

Aim 3: Determine optimal biomarker cutpoints; months 43-48.

- a. Perform statistical analyses using semi-parametric modeling followed by preparation of ROC curves using HPFS data. (Hopkins)
- b. Apply cutpoints from above to the two other cohorts validation. (Hopkins)
- c. Present findings at national cancer meeting and IMPaCT meeting. (Hopkins and Harvard)
- d. Write manuscript. (Hopkins and Harvard)
- e. Prepare final report to DOD. (Hopkins)
- f. Attend IMPaCT meeting. (Hopkins and Harvard)