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14. ABSTRACT Three independent sets of normal breast tissues without evidence of cancer, either obtained from patients undergoing reduction mammoplasty or in women at time of autopsy, have been analyzed. The postdoctoral trainee has shown that moderate to dramatic telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal terminal ductal lobular units. However, the extent and degree of telomere shortening varies by the individual. These data imply that there is a reservoir of genetically altered, yet histologically normal, cells within normal breast tissues that may represent fertile ground for tumor development. Since telomere shortening has been associated with cellular senescence and dysfunctional telomeres have been linked to the DNA damage response pathway in cancerous tissues, ongoing experiments are assessing senescence-associated markers and DNA damage response pathway markers in histologically normal human breast tissues that display either normal or short telomeres (i.e. prior to tumor formation). In addition, the proposed investigation has provided grounding in both basic and translational breast cancer research for the trainee. The interactive, multidisciplinary research environment at Johns Hopkins has provided the investigator opportunities to interact with pathologists, oncologists and epidemiologists, thus fostering future success as an independent translational breast cancer researcher.					
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INTRODUCTION

The overall goal of our research is to determine the role telomere biology plays in the initiation and progression of human breast cancer. Independent investigations, including from our own laboratory, have demonstrated the existence of cells with shortened telomeres in histologically normal tissues (Meeker *et al*, 2004; Kurabayashi *et al*, 2008). In this proposal, we are characterizing the cellular consequences of these telomere shortened normal cells. Since telomere shortening has been associated with cellular senescence and dysfunctional telomeres have been associated with activation of the DNA damage response pathway in tumor tissues, including premalignant lesions, we are assessing senescence-associated markers (Specific Aim #1) and DNA damage response pathway markers (Specific Aim #2) in histologically normal human breast tissues that display either normal or short telomeres (*i.e.* prior to tumor formation). Furthermore, the normal cellular response to senescence and activation of DNA damage response pathway is being monitored by artificially shortening telomeres in human mammary epithelial cells isolated from primary tissues (Specific Aim #3). In addition to the scientific investigations, this award has provided the trainee opportunities to interact with pathologists, oncologists, and epidemiologists to learn (i) normal and abnormal breast morphology, (ii) the strengths and limitations of currently used breast cancer biomarkers, (iii) current standards of breast cancer treatment, and (iv) the scientific rationale for ongoing clinical trials. These interactions are helping to foster future success as an independent translational breast cancer researcher.

BODY

Summary of timeline: This BCRP Postdoctoral Training Award was initiated with a September 1, 2009 start date. Since the proposal included the use of human subjects, we wrote and received approval from the Office of Human Subjects Research Institutional Review Board at Johns Hopkins (November 12, 2009) and from the Human Research Protection Office of the U.S. Army Medical Research and Materiel Command (January 27, 2010) for collection of the clinical samples to be used in this investigation. Due to unexpected delays in tissue acquisition for Specific Aims #1 and #2 and in establishing the lentiviral vector outlined in Specific Aim #3, a one year no-cost extension was requested (June 19, 2012) and was recently granted (June 29, 2012).

Tissue Collection:

During Year 1, collection protocols for clinical specimens were established for fluorescent *in situ* hybridization (FISH), immunofluorescence (IF) and immunohistochemistry (IHC) experiments that utilize formalin-fixed, paraffin-embedded (FFPE) tissues. Protocols were also established for primary cell culture experiments that utilize freshly collected human breast tissue. During Year 1, histologically normal breast tissue from 1cm and 5cm away from the visible tumor margin was obtained from 27 women undergoing radical mastectomy. Additionally, histologically normal breast tissue from the right and left breast was obtained from 14 women undergoing bilateral reduction mammoplasty. During Year 2, histologically normal breast tissue from 1cm and 5cm away from the visible tumor margin was obtained from an additional 21 women undergoing radical mastectomy. Additionally, histologically normal breast tissue from the right and left breast was obtained from an additional 6 women undergoing bilateral reduction mammoplasty.

During Year 3, the collection of these tissues continued and to date, histologically normal breast tissues (1cm and 5cm away from the visible tumor margin) have been obtained from a total of 58 women undergoing radical mastectomy. Likewise, histologically normal breast tissues from the right and left breast have been obtained from a total of 23 women undergoing bilateral reduction mammoplasty. For all of these specimens, FFPE tissue blocks have been generated. In addition, using published protocols (*Speirs et al, 1998*), primary cell cultures have been established from 24 of the women undergoing radical mastectomy and from 8 women undergoing reduction mammoplasty.

Results: Using the FFPE specimens obtained from the 23 reduction mammoplasty specimens outlined above, telomere lengths were determined using the telomere-specific FISH assay developed in our laboratory. As shown in Figure 1, telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in histologically normal terminal ductal lobular units (TDLU). In some TDLUs, the luminal cells, negative for smooth muscle actin (SMA), show comparable telomere intensities similar to the adjacent myoepithelial cells (panel A). In contrast, some TDLUs demonstrate dim telomere signals in the luminal cells when compared to the adjacent myoepithelial cells (panel B). Through digital image analysis, quantitative determination of the telomere FISH signals confirms this moderated telomere shortening (panel C). Strikingly, telomere shortening occurs in the majority of histologically normal TDLUs analyzed from patients undergoing reduction mammoplasty, but the extent and degree of shortening varies by the individual.

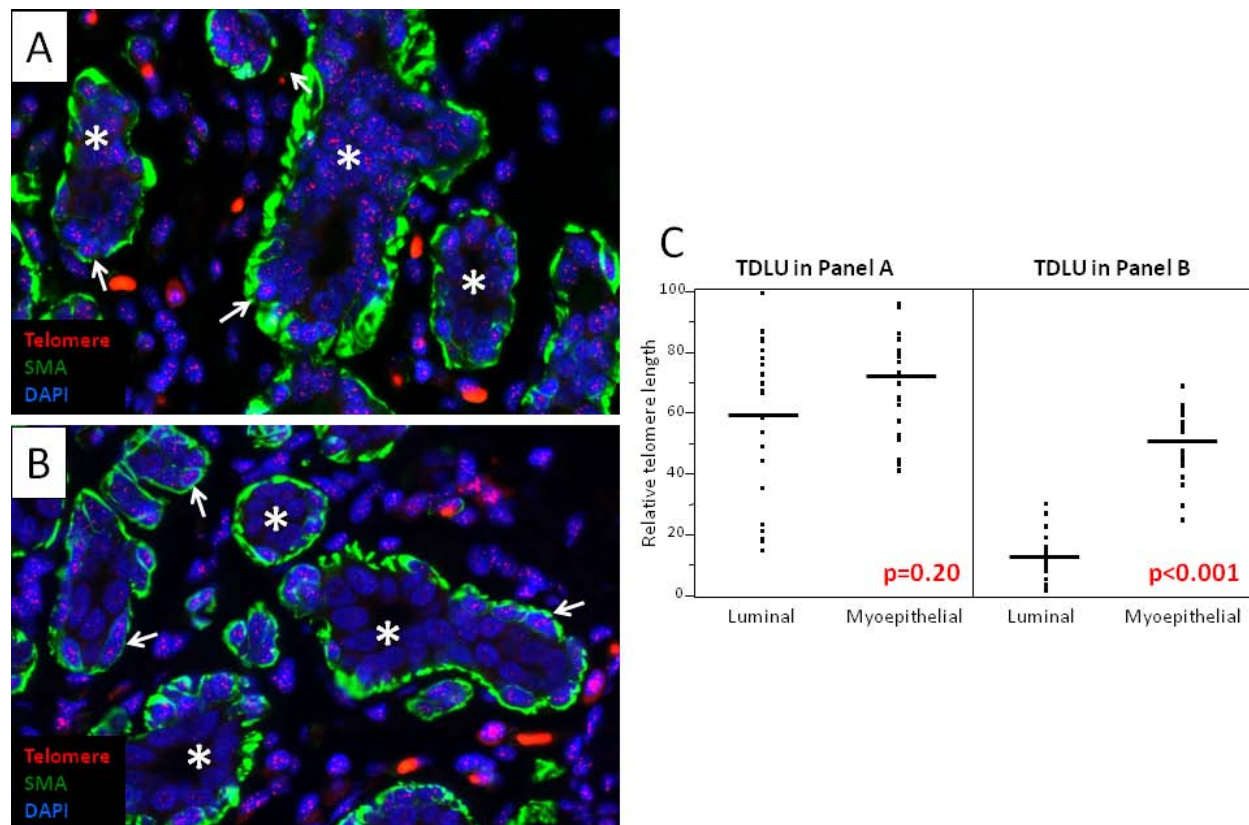


Figure 1. Telomere-specific FISH in normal breast tissues obtained from women undergoing reduction mammoplasty surgeries. (A) A normal breast TDLU with normal length telomeres in all cell types present. (B) A normal breast TDLU with short telomeres in the luminal cells. The asterisks (*) show luminal cells and the white arrows show myoepithelial cells demarcated by the presence of smooth muscle actin (green). Telomeres (red) and DAPI-stained nuclei (blue) are also shown. (C) Quantification by digital image analysis of relative telomere lengths by determining the mean DAPI-normalized telomere signal intensities in 25 randomly selected luminal and myoepithelial cells.

Since telomere shortening has been linked to age and all the women in the reduction mammoplasty cohort were relatively young, we sought to assess another cohort of normal breast tissues obtained from women. To accomplish this, we collaborated with Dr. Mark Sherman (Division of Cancer Epidemiology & Genetics; National Cancer Institute) to obtain normal breast tissues from 7 women at the time of autopsy. As observed in the previous cohort, telomere shortening occurred in the majority of histologically normal TDLUs analyzed from these women; again, the extent and degree of shortening varied by the individual (Figure 2).

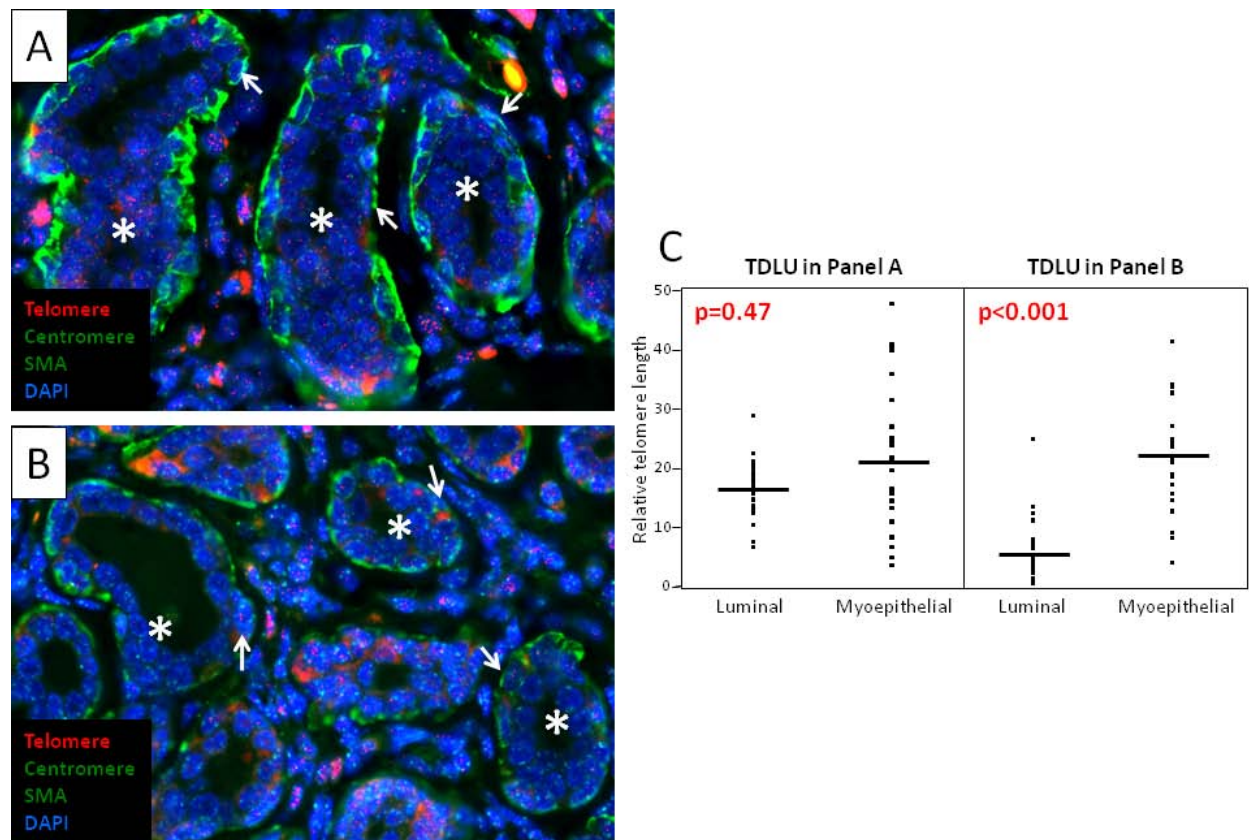


Figure 2. Telomere-specific FISH in normal breast tissues obtained from a woman at time of autopsy. (A) A normal breast TDLU with normal length telomeres in all cell types present. (B) An adjacent normal breast TDLU with short telomeres in the luminal cells. The asterisks (*) show luminal cells and the white arrows show myoepithelial cells demarcated by the presence of smooth muscle actin (green). Telomeres (red), centromeres (green) and DAPI-stained nuclei (blue) are also shown. (C) Quantification by digital image analysis of relative telomere lengths

by determining the mean DAPI-normalized telomere signal intensities in 25 randomly selected luminal and myoepithelial cells.

Finally, we sought to validate our findings in a third, independent cohort of normal breast tissues obtained from women without evidence of cancer. To accomplish this, we collaborated with Dr. Kala Visvanathan (Department of Epidemiology; The Johns Hopkins Bloomberg School of Public Health) to obtain normal breast tissues from eleven women obtained by reduction mastectomy. As observed in the previous two sets, telomere shortening occurred in the majority of histologically normal TDLUs analyzed from these women; again, the extent and degree of shortening varied by the individual. Results from the three independent sets are summarized in Table 1.

Table 1. Summary of the three independent sets of normal breast tissue with regards to the presence of telomere shortening in the luminal epithelial cell compartment.

Set	Tissue Type	N	# of cases with luminal telomere shortening (% of all cases)
Hopkins	Reduction Mammoplasty	23	23 (100%)
NCI	Autopsy	7	7 (100%)
Bloomberg SoPH	Reduction Mammoplasty	11	11 (100%)

In summary, moderate to severe telomere shortening is highly prevalent within histologically normal TDLUs obtained from women undergoing reduction mammoplasty surgeries and in women at time of autopsy. The dramatic telomere shortening specifically occurs in luminal epithelial cells, but not in myoepithelial cells. All women examined in the 3 independent sets contained some luminal telomere shortening in their normal TDLUs, but the extent and degree of luminal telomere shortening varied by the individual. These data were presented as a poster presentation at the 2011 AACR Breast Cancer Research Meeting (Appendix A) and will be included in a manuscript (in preparation).

Since the overall goal of our research is to determine the role telomere biology plays in the initiation and progression of human breast cancer, in addition to the ongoing studies in normal, cancer-free breast tissues, we have also evaluated telomere lengths in breast tumors. Telomere lengths were evaluated in invasive breast cancer cases (N=103) and the presence of short cancer cell telomere lengths were associated with the more aggressive breast cancer subtypes, (eg. HER-2 positive and triple-negative tumors), suggesting tumor telomere length may have clinical utility as a prognostic and/or risk biomarker (*Heaphy et al, Modern Pathology, 2011*).

Dysfunctional telomeres cause genomic instability via chromosomal breakage-fusion-bridge cycles. In the majority of human cancers, telomere dysfunction is attenuated through up-regulation of the enzyme telomerase. However, telomere loss may also be compensated in some cancers by the telomerase-independent telomere maintenance mechanism termed alternative lengthening of telomeres (ALT). The ALT phenotype has rarely been reported in epithelial malignancies; however, our laboratory previously reported the presence of ALT in a small subset of invasive breast carcinomas (*Subhawong et al, 2009*). We confirmed this finding by assessing a total of 377 breast carcinomas and observed the ALT phenotype in 7 cases (2%). In addition to

the breast data, we comprehensively surveyed the ALT phenotype in 6,110 primary tumors from 94 different human cancer subtypes. Overall, the prevalence of the ALT phenotype was 3.73%; however, the prevalence varied vastly between different subtypes. Since ALT-positive cancers are predicted to be resistant to anti-telomerase therapies, these findings may have therapeutic implications (*Heaphy et al, American Journal of Pathology, 2011*).

Since the ALT pathway plays a critical role in tumorigenesis in certain tumor types, it was interesting to our group that two genes, *ATRX* and *DAXX*, that participate in chromatin remodeling at telomeres were found to be mutated at a high rate in pancreatic neuroendocrine tumors (PanNETs); a tumor type that contains a high proportion of tumors displaying the ALT phenotype (*Jiao et al, 2011*). In collaboration with this group, breast tumor genomic DNA (N=96) was sequenced for *ATRX* and *DAXX*. Unfortunately, we did not observe any mutations in these two genes. However, we did observe that all of the PanNETs that exhibited the ALT phenotype had *ATRX* or *DAXX* abnormalities. Subsequent sequencing of *ATRX* and *DAXX* in other cancers revealed *ATRX* mutations in 1.5-14.3% of various tumors of the central nervous system, and these mutations occurred only in tumors exhibiting ALT. Therefore, we concluded that alterations in *ATRX* and *DAXX* are associated with the ALT phenotype in human cancers (*Heaphy et al, Science, 2011*). This investigation was presented at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Fellow Research Day as a poster presentation and the trainee was awarded 1st place for Basic Science (Appendix B).

In addition to the outlined scientific investigations, the trainee has received experimental training in numerous methods including: fluorescence *in situ* hybridization, immunostaining, histopathology, primary cell culture, study design and statistical analysis. The trainee has also interacted and collaborated with oncologists, surgeons, pathologists, molecular epidemiologists and other Ph.D. research scientists who specialize in the research and treatment of breast cancer. The trainee has attended weekly journal clubs, Oncology translational research seminars, breast cancer seminars, Pathology Grand Rounds, specific meetings of the Hopkins Breast SPORE program and “sign-out” sessions with surgical breast pathologists. Finally, the trainee was invited to write a review article describing “The potential utility of telomere-related markers for cancer diagnosis” (*Heaphy et al, Journal of Cellular and Molecular Medicine, 2011*).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that dramatic telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal TDLUs from women free of cancer undergoing reduction mammoplasty and in women at time of autopsy without evidence of cancer.
- Demonstrated that the extent and degree of telomere shortening in histologically normal TDLUs varies by the individual.
- Demonstrated that telomere lengths were shorter in the more aggressive breast cancer subtypes, suggesting tumor telomere length may have clinical utility as a prognostic and/or risk stratification biomarker for breast cancer.

- Determined the prevalence of the ALT phenotype in breast carcinoma (2%) and comprehensively surveyed the prevalence of the ALT phenotype in 6,110 primary tumors from a broad range of human cancer subtypes (3.73%).
- Demonstrated that alterations in two genes, *ATRX* and *DAXX*, which participate in chromatin remodeling at telomeres are closely associated with the ALT phenotype in human cancers.

REPORTABLE OUTCOMES

Peer reviewed manuscripts (during reporting period):

None published during this reporting period.

Published Abstracts at National Meetings (during reporting period):

C.M. Heaphy, M.E. Sherman, B.K. Vonderhaar, P. Argani and A.K. Meeker. Significant telomere shortening is common in luminal epithelial cells in histologically normal breast tissues from women without cancer. AACR Special Meeting: Advances in Breast Cancer Research. San Francisco, CA. October, 2011.

Awards (during reporting period):

1st Place for Basic Research in the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Fellow Research Day for a poster presentation titled “Altered telomeres in tumors with *ATRX* and *DAXX* mutations”. May, 2012.

CONCLUSIONS

Through this training grant, generated data generated have been have been presented at numerous national meetings. Importantly, the postdoctoral trainee is a first author on a number of manuscripts published in high-profile journals (eg. *Science* and *The American Journal of Pathology*). Another manuscript was published in *Modern Pathology*; and, an invited review article assessing the potential utility of telomere-related markers in the field of cancer diagnosis was published in the *Journal of Cellular and Molecular Medicine*. The investigator is progressing with all of his educational and training goals.

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Significant telomere shortening is common in luminal epithelial cells in histologically normal breast tissues from women without cancer

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Telomeres are nucleoprotein complexes comprised of the hexanucleotide DNA repeat sequence, TTAGGG, and numerous telomere-associated proteins, including the six member Shelterin complex. The telomere complex primarily functions to mask double strand break DNA damage signals at telomeres, inhibit exonucleolytic degradation, and prevent chromosomal fusions. However, through multiple mechanisms, telomeres can become dysfunctional. In normal somatic cells, significant telomere shortening leads to p53-dependent senescence or apoptosis. In cancer cells, these cell cycle checkpoints are abrogated, and if unchecked cellular proliferation continues, then genomic instability may ensue via chromosomal breakage-fusion-bridge cycles initiated by critically short telomeres. Numerous investigations have shown that telomere shortening is present in the majority of mammary carcinomas, both at the *in situ* and invasive stages. Interestingly, telomere shortening has been observed in a subset of histologically normal terminal ductal lobular units (TDLU), primarily in cancer-bearing women, but this observation has not been fully characterized. Here, we assessed the prevalence and degree of telomere shortening in histologically normal breast tissues. Telomere lengths were assessed directly at the single cell level by fluorescence *in situ* hybridization in breast tissues obtained from women without breast cancer, undergoing reduction mammoplasty surgeries and from women at the time of autopsy. Strikingly, moderate to severe telomere shortening is highly prevalent within the luminal epithelial cells in histologically normal TDLUs. All women contained telomere shortening in a subset of their normal appearing TDLUs, although the extent and degree of luminal telomere shortening varied by the individual. This finding has potential to illuminate the mechanisms that underpin breast cancer initiation. Assessment of these early molecular alterations is critical in providing unique insights that may lead to new strategies for early prevention, risk assessment or even the development of new treatment modalities.

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(Pathology / Prostate Cancer)

Title: **ALTERED TELOMERES IN TUMORS WITH *ATRX* AND *DAXX* MUTATIONS**

Abstract: Recent exomic sequencing of pancreatic neuroendocrine tumors (PanNETs) has revealed frequent inactivating mutations of the *ATRX* and *DAXX* genes. The products of these genes have been shown to localize at heterochromatic sites, including telomeres, prompting us to assess telomere status in PanNETs harboring these mutations. We found that 25/41 (61%) of PanNETs examined displayed evidence of Alternative Lengthening of Telomeres (ALT), a telomerase-independent telomere maintenance mechanism found in cancers that have not activated telomerase. All 25 ALT-positive cases exhibited alterations of *ATRX* or *DAXX* detectable by sequencing (21 cases) or by immunolabeling (4 cases), whereas the 16 ALT-negative cases had no alterations in either gene. To determine whether this 100% association was generalizable, we determined the sequence of *ATRX* and *DAXX* in 447 cancers from various sites. We found mutations most commonly in pediatric glioblastoma multiformae (GBM) (11.1%), adult GBM (7.3%), oligodendrogliomas (7.7%) and medulloblastomas (1.5%); and showed that ALT perfectly correlated with somatic mutations of *ATRX*. Finally, we showed that there was a deletion of *ATRX* in the prototypical cell line U-2 OS, used to define the ALT phenotype in telomerase-negative cancer-derived cell lines. These data suggest that an alternative telomere maintenance function may operate in human tumors with alterations in the *ATRX* or *DAXX* genes.