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14. ABSTRACT
The investigator has shown that moderate telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal terminal ductal lobular units analyzed from patients undergoing reduction mammoplasty and in women at time of autopsy. 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 designed to assess the 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 has provided the investigator opportunities to interact with pathologists and oncologists, thus fostering future success as an independent breast cancer researcher. To date, all tasks, as outlined in the Statement of Work, are on schedule.

15. SUBJECT TERMS
Breast cancer, Cellular senescence, DNA damage, Telomere, Terminal ductal lobular unit

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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 and oncologists to learn normal and abnormal breast morphology, the strengths and limitations of currently used breast cancer biomarkers, current standards of breast cancer treatment, and the scientific rationale for ongoing clinical trials. To date, all tasks, as outlined in the Statement of Work, are on schedule.

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. During Year 1, collection protocols for clinical specimens were established for fluorescent *in situ* hybridization (FISH) and immunofluorescence (IF) and/or 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, 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 48 women undergoing radical mastectomy. In addition, histologically normal breast tissues from the right and left breast have been obtained from a total of 20 women undergoing bilateral reduction mammoplasty. For all of these specimens, FFPE tissue blocks have been generated. Additionally, using published protocols (*Speirs et al, 1998*), primary cell cultures have been established from 21 of the women undergoing radical mastectomy and from 6 women reduction mammoplasty.

Results: Using the FFPE specimens obtained from 14 of the 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 (Fig. 1A). In contrast, some TDLUs demonstrate weak telomere signals in the luminal cells when compared to the adjacent myoepithelial cells (Fig. 1B). Quantitative analysis of the telomere FISH signals confirms this dramatic telomere shortening (Fig. 1C).

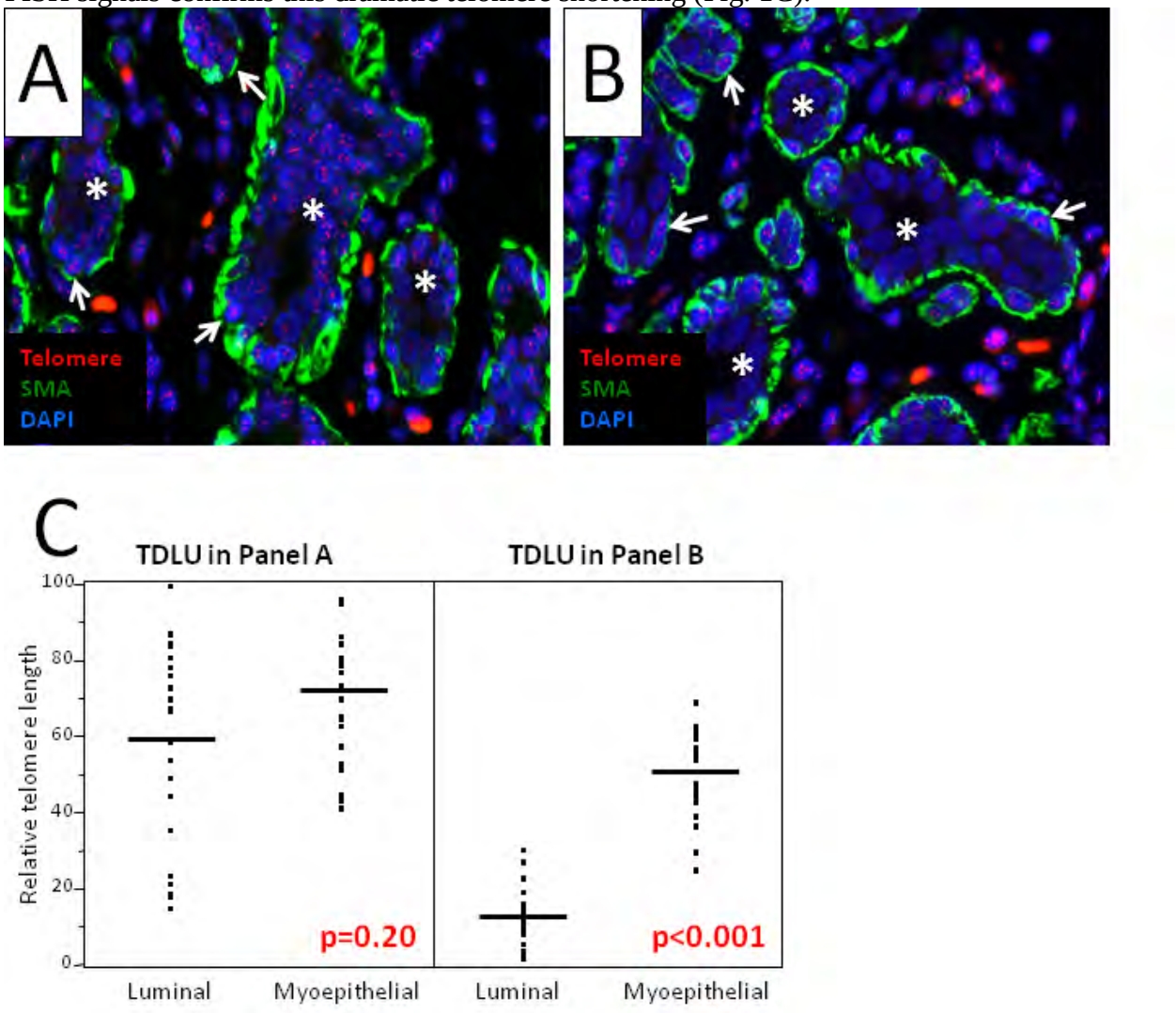

Figure 1. *Fluorescence in situ hybridization (FISH) analysis of telomere lengths in normal breast tissues obtained from patients undergoing reduction mammoplasty surgeries (**A**) A normal breast terminal ductal lobular unit (TDLU) with normal length telomeres in all cell types present (Case 7). (**B**) A normal breast TDLU with short telomeres in the luminal cells (Case 2). 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.*

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 (Table 1).

Table 1. Presence of telomere shortening in luminal epithelial cells within histologically normal TDLUs in breast tissues obtained from fourteen women undergoing reduction mammoplasty surgeries.

Case (side of breast)	Luminal telomere shortening?	# TDLUs with short telomeres	Total # of TDLUs	% TDLUs with short telomeres
1 (left)	yes	20	26	77%
1 (right)	yes	19	23	83%
2 (left)	yes	22	22	100%
2 (right)	yes	25	26	96%
3 (left)	yes	4	8	50%
3 (right)	yes	4	5	80%
4 (left)	yes	19	21	90%
4 (right)	yes	19	20	95%
5 (left)	yes	2	2	100%
5 (right)	yes	1	3	33%
6 (left)	yes	21	23	91%
6 (right)	yes	16	18	89%
7 (left)	yes	5	8	63%
7 (right)	yes	8	27	30%
8 (left)	yes	7	7	100%
8 (right)	yes	12	19	63%
9 (left)	*Fibroadenoma*			
9 (right)	*Extensive fibrocystic changes*			
10 (left)	yes	3	22	14%
10 (right)	yes	6	18	33%
11 (left)	yes	7	24	29%
11 (right)	yes	11	27	41%
12 (left)	no	0	4	0%
12 (right)	yes	13	21	62%
13 (left)	yes	5	5	100%
13 (right)	yes	9	9	100%
14 (left)	yes	2	3	67%
14 (right)	*Only stroma & adipose tissue*			

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 seven 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 (Table 2).

Table 2. Presence of telomere shortening in luminal epithelial cells within histologically normal TDLUs in breast tissues obtained from seven women at time of autopsy.

Case (side of breast)	Luminal telomere shortening?	# TDLUs with short telomeres	Total # of TDLUs	% TDLUs with short telomeres
1 (left)	yes	17	23	74%
1 (right)	yes	8	18	44%
2 (left)	yes	1	1	100%
2 (right)	*Only stroma & adipose tissue*			
3 (left)	yes	2	18	11%
3 (right)	*Only stroma & adipose tissue*			
4 (left)	yes	4	12	33%
4 (right)	yes	10	17	59%
5 (left)	yes	17	24	71%
5 (right)	yes	14	27	52%
6 (left)	yes	4	10	40%
6 (right)	yes	13	15	87%
7 (left)	yes	2	3	67%
7 (right)	*Only stroma & adipose tissue*			

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 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 poster presentations at the 2011 Era of Hope Meeting in Orlando, FL (Appendix A) and at the 4th Annual Safeway Breast Cancer Retreat: Bench to Bedside to Population in Baltimore, MD (Appendix B).

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 breast tissues, we have also evaluated telomere lengths in breast tumors. Using the telomere-specific FISH, we evaluated telomere lengths in 103 cases of invasive breast cancer and correlated the telomere lengths with other established molecular markers. This study demonstrated that telomere lengths in the cancer cells were shorter in the more aggressive breast cancer subtypes, such as luminal B, HER-2 positive and triple-negative tumors (compared to luminal A tumors), suggesting tumor telomere length may have clinical utility as a prognostic and/or risk marker for breast cancer. The data from this investigation was published in ***Modern Pathology*** (Appendix C).

During malignant transformation, genomic instability ensues via chromosomal breakage-fusion-bridge cycles caused by eroded, dysfunctional telomeres. 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), which is dependent on homologous recombination. The ALT phenotype has rarely been reported in epithelial malignancies; however, our laboratory recently reported the presence of ALT in a small subset of invasive breast carcinomas (*Subhawong et al, 2009*). To confirm and extend upon this finding, we assessed 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 two independent sets of fixed specimens, comprising 6,110 primary tumors from 94 different human cancer subtypes, 541 benign neoplasms and 264 normal tissue samples. Overall, the prevalence of the ALT phenotype was observed in 3.73% (228/6,110) of all tumor specimens; however, the prevalence varied vastly between different subtypes. Importantly, the ALT phenotype was not observed in benign neoplasms or normal tissues. These findings may have therapeutic consequences, since ALT-positive cancers are predicted to be resistant to anti-telomerase therapies. The data from this investigation was recently published in ***The American Journal of Pathology*** (Appendix D). Prior to publication, the trainee presented these findings at the United States and Canadian Academy of Pathology (USCAP) Annual Meeting and the poster presentation was awarded the prestigious Stowell-Orbison Award (Appendix E). In addition, the project was awarded the Excellence in Translational Research at the 13th Annual Johns Hopkins Department of Pathology Young Investigator's Day (Appendix F).

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; a tumor type that contains a high proportion of tumors displaying the ALT phenotype (*Jiao et al, 2011*). Genomic DNA from 96 breast carcinomas was sequenced for *ATRX* and *DAXX*. Unfortunately, we did not observe any mutations in these two genes within this set of tumors; although, the ALT status for these cases was unknown. However, we did observe that all of the pancreatic neuroendocrine tumors 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% to 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. This investigation was recently published in ***Science*** (Appendix G).

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 for the ***Journal of Cellular and Molecular Medicine*** describing "The potential utility of telomere-related markers for cancer diagnosis" (Appendix H).

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 patients undergoing reduction mammoplasty and in women at time of autopsy.

- 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, such as luminal B, HER-2 positive and triple-negative tumors, suggesting tumor telomere length may have clinical utility as a prognostic and/or risk marker 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:

C.M. Heaphy, A.P. Subhawong, A. Gross, Y. Konishi, N. Kouprina, P. Argani, K. Visvanathan, and A.K. Meeker. Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes. Modern Pathology, 24:194-200, 2011.

C.M. Heaphy and A.K. Meeker. The potential utility of telomere-related markers for cancer diagnosis. Journal of Cellular and Molecular Medicine, 15:1227-1238, 2011. *Invited review*

C.M. Heaphy, A.P. Subhawong, S-M. Hong, M.G. Goggins, E.A. Montgomery, E. Gabrielson, G.J. Netto, J.I. Epstein, T.L. Lotan, W.H. Westra, I-M. Shih, C.A. Iacobuzio-Donahue, A. Maitra, Q.K. Li, C.G. Eberhart, J.M. Taube, D. Rakheja, R.J. Kurman, T. Wu, R.B. Roden, P. Argani, A.M. De Marzo, L. Terracciano, M. Torbenson and A.K. Meeker. Prevalence of the Alternative Lengthening of Telomeres (ALT) telomere maintenance mechanism in human cancer subtypes. The American Journal of Pathology, 179:1608-1615, 2011.

C.M. Heaphy*, R.F. de Wilde*, Y. Jiao*, A.P. Klein, B.H. Edil, C. Shi, C. Bettegowda, F.J Rodriguez, C.G Eberhart, S. Hebbar, G.J. Offerhaus, R. McLendon, B.A. Rasheed, Y. He, H. Yan, D.D. Bigner, S.M. Oba-Shinjo, S.K. Nagahashi Marie, G.J. Riggins, K.W. Kinzler, B. Vogelstein, R.H. Hruban, A. Maitra, N. Papadopoulos and A.K. Meeker. Altered telomeres in tumors with *ATRX* and *DAXX* mutations. Science, 333:425, 2011.
**Authors contributed equally to this study*

Published Abstracts at National Meetings:

C.M. Heaphy, A.P. Subhawong, S. Hong, M. Goggins, E. Montgomery, E. Gabrielson, G. Netto, J.I. Epstein, T.L. Lotan, W. Westra, I. Shih, C. Iacobuzio-Donahue, A. Maitra, Q. Li, C. Eberhart, J. Taube, R. Kurman, T Wu, R. Roden, P. Argani, A.M. De Marzo, L. Terracciano, M. Torbenson and A.K. Meeker (2011). Prevalence of Alternative Lengthening of Telomeres (ALT) in human cancer subtypes. United States and Canadian Academy of Pathology (USCAP) Annual Meeting. San Antonio, TX.

C.M. Heaphy, M.E. Sherman, B.K. Vonderhaar, P. Argani and A.K. Meeker (2011) Cellular Consequences of Telomere Shortening in Histologically Normal Breast Tissues. Era of Hope DoD Conference, Orlando, FL.

Awards:

Stowell-Orbison Award at the United States and Canadian Academy of Pathology (USCAP) Annual Meeting for poster presentation titled “Prevalence of Alternative Lengthening of Telomeres (ALT) in Human Cancer Subtypes”.

The 13th Annual Johns Hopkins Department of Pathology Young Investigator’s Day Award for Excellence in Translational Research for poster presentation titled “Prevalence of Alternative Lengthening of Telomeres (ALT) in Human Cancer Subtypes”.

CONCLUSIONS

To date, all tasks; as outlined in the Statement of Work are on schedule. Tissue collection protocols have been established and procurement of clinical samples is ongoing. The accrual rates for obtaining tissues from radical mastectomies and reduction mammoplasties are on target. Data generated from this training grant have been have been presented at national meetings and the 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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Cellular Consequences of Telomere Shortening in Histologically Normal Breast Tissues

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Background: Telomeres, nucleoprotein complexes located at the extreme ends of eukaryotic chromosomes, function to mask double strand break DNA damage signals, inhibit exonucleolytic degradation, and prevent chromosomal fusions. In normal somatic cells, critically short, dysfunctional telomeres are prone to chromosome fusions and breakages which lead to p53-dependent senescence or apoptosis. However, these mechanisms are inactivated in cancer cells, allowing chromosomal instability. Numerous investigations have shown that telomere shortening is present in the majority of mammary carcinomas, both at the *in situ* and invasive stages. Interestingly, moderate telomere shortening also occurs in a subset of histologically normal terminal ductal lobular units (TDLU). Additionally, telomere shortening and genomic instability occur in histologically normal breast tissues adjacent to the corresponding tumors at least 1cm from the visible tumor margins. In all, these data imply that there is a reservoir of genetically altered, yet histologically normal, cells within normal breast tissues that may represent a fertile ground for tumor development.

Objective: To assess the prevalence and degree of telomere shortening in normal cells and characterize the molecular pathways (cellular senescence and DNA damage response) in histologically normal breast tissues that display either normal or short telomeres (*i.e.* prior to tumor formation).

Methodology: Our laboratory developed a combined fluorescence *in situ* hybridization (FISH)/immunostaining assay that allows simultaneous telomere length assessment and the localization of proteins in specific cells within a tissue section. Using this assay, protein expression patterns of senescence-associated markers and markers of the DNA damage response pathway are assessed in cells with different telomere lengths within normal tissues obtained from reduction mammoplasties and in matched histologically normal tissues at defined distances (1cm and 5cm) from the visible tumor margin.

Results: Strikingly, moderate to severe telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal terminal ductal lobular units analyzed from patients undergoing reduction mammoplasty, but the extent and degree of shortening varies by the individual. Additionally, luminal telomere shortening is observed in histologically normal tissues 1cm and 5cm from visible tumor margins in specimens obtained by radical mastectomy. Protein expression patterns for markers of cellular senescence and the DNA damage response pathway will be discussed.

Conclusions and Impact: The novel finding that telomeres shorten in a subset of histologically normal TDLUs has tremendous potential to illuminate the mechanisms that underpin the initiation of breast cancer. Assessment of these early molecular events 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.

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.

Modern Pathology (2011) 24, 194–200

Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes

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Telomeres are nucleoprotein structures that protect chromosome ends from degradation and recombination. Cancers often have critically shortened telomeres, contributing to genomic instability. Many of these tumors activate telomerase to stabilize telomeric ends and achieve a capacity for unlimited replication. Telomere shortening has been reported in *in situ* and invasive carcinomas, including breast, and has been associated with disease recurrence after surgical resection. However, previous studies have not evaluated breast cancer subtypes. The objective of this study was to evaluate telomere lengths in different subtypes of breast cancer. Breast carcinomas ($n=103$) identified between 2001 and 2010 from patients seen at the Johns Hopkins Hospital were categorized into luminal A ($n=18$), luminal B ($n=28$), HER-2-positive ($n=20$) and triple-negative carcinomas ($n=37$) based on tumor characteristics. Telomere lengths were assessed directly at the single cell level by fluorescence *in situ* hybridization, and patient groups were compared using Fisher's exact tests. ER-negative status ($P=0.022$), PR-negative status ($P=0.008$), HER-2-positive status ($P=0.023$) and p53-positive status ($P=0.022$) were associated with shorter telomere length. A larger proportion of luminal A cancers had normal or long telomere lengths as compared with luminal B cases ($P=0.002$), HER-2-positive cases ($P=0.011$) or triple-negative cases ($P=0.0003$). Luminal B, HER-2-positive and triple-negative cases did not differ significantly. Telomere length was shorter in more aggressive subtypes, such as luminal B, HER-2-positive and triple-negative tumors, suggesting that tumor telomere length may have utility as a prognostic and/or risk marker for breast cancer.
Modern Pathology (2011) **24,** 194–200; doi:10.1038/modpathol.2010.198; published online 5 November 2010

Keywords: breast cancer; HER-2; luminal A; luminal B; subtype; telomere; triple-negative

Telomeres, specialized nucleoprotein structures, function to protect and stabilize the ends of eukaryotic chromosomes by preventing chromosome fusions, exonucleolytic degradation and masking telomere-induced double-strand DNA break damage signals. However, telomeres can become critically shortened, and dysfunctional, by several mechanisms including incomplete replication, wherein they are shortened during each cycle of chromosome replication.[1] In normal cells, telomere shortening induces tumor-suppressive checkpoint pathways, such as cellular senescence or apoptosis, which halts cell-cycle progression before telomeres become destabilized.[2,3] However, abrogation of these checkpoints leads to continued cell division. Consequently, there is a limit to the number of doublings somatic cells can undergo before triggering successive rounds of chromosome breakage–fusion–bridge cycles, thus driving chromosome amplification and loss of structural rearrangements.[4]

It is well established that telomere shortening is present in the majority of *in situ* and invasive carcinomas,[5] including breast.[6,7] Thus, telomere shortening is an early event in malignant transformation. In addition to tumor initiation, short dysfunctional telomeres may also affect disease progression. Previous studies have shown reduced telomere length in grade III tumors,[8] and reduced telomere DNA content, a surrogate for telomere

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length, correlates with aneuploidy and lymph node metastasis,[9] and shorter telomeres were associated with higher stage and histological grade.[10] A retrospective study ($n=77$) showed that short telomeres were associated with tumor size, nodal involvement, TNM stage and was an independent predictor of 5-year overall survival and 5-year breast cancer-free survival.[11] A larger population-based prospective study ($n=530$) showed that short telomeres conferred a relative hazard of breast cancer recurrence of 2.88, after adjusting for prognostic factors and adjuvant therapies.[12] However, none of these studies took into consideration specific breast cancer subtypes that are now used to help guide treatment decisions.

Four main molecular classes of breast cancer were first identified by gene expression profiling.[13] On further validation, these subtypes correlate well with the clinical characterization of ER, PR and HER-2 protein expression status.[14,15] These groups are luminal A carcinomas (ER and PR positive, HER-2 negative), luminal B carcinomas (ER or PR positive, HER-2 positive), HER-2 carcinomas (ER and PR negative, HER-2 positive) and triple-negative carcinomas (ER/PR/HER-2 negative). Important for clinical care, these subtypes predict prognosis and therapeutic response.[16–18] Luminal A tumors respond well to selective estrogen receptor modulators, such as tamoxifen.[19] Luminal B tumors tend to be less sensitive to hormonal therapies and thus carry a worse prognosis than do luminal A tumors. HER-2-positive cancers tend to be high grade, aggressive and carry a poor prognosis; however, they respond well to trastuzumab, an anti-HER-2 monoclonal antibody. Conversely, triple-negative carcinomas are extremely aggressive and currently do not respond to any hormonal or antibody-based targeted therapy.[20]

The objective of this study was to evaluate telomere lengths in subtypes of breast carcinoma. This was accomplished using a fluorescence *in situ* hybridization (FISH) assay that allows telomere length assessments in formalin-fixed, paraffin-embedded archival material, while providing single-cell resolution and keeping the tissue architecture intact. We evaluated telomere length in 103 cases of invasive breast cancer and correlated telomere length with established molecular markers.

Materials and methods

Case Selection

All of the 103 cases evaluated in this study were incident breast carcinomas that were surgically resected at the Johns Hopkins Hospital from 2001 to 2010. At the time of resection, specimens were freshly sectioned, fixed overnight in 10% neutral buffered formalin and uniformly processed. Women treated with neoadjuvant chemotherapy were not included in this study. Clinical characteristics, such as age at diagnosis, weight, ethnicity, parity status and menopausal status were obtained from patient's medical records. Pathological characteristics, such as Elston grade and TNM stage, were obtained from patient's pathology records. This study was approved by the Institutional Review Board of the Johns Hopkins School of Medicine.

Tissue Microarray Construction

For the majority of cases ($n=72$), tissue microarrays were constructed as described previously.[21,22] In brief, each tissue microarray consisted of 99 tissue cores, each 1.4 mm in diameter. These cores were arranged in 9 rows and 11 columns. Column 6 consisted of various unrelated control tissues, leaving 90 cores on the array for breast carcinoma samples. For each carcinoma case, 5 areas were identified on the hematoxylin and eosin slides, punched from the corresponding donor blocks and placed on the array. Among the five samples of each case, we attempted to include the normal tissue and carcinoma *in situ* in one sample if possible, leaving four to five cores of invasive ductal carcinoma per case. Any case that displayed a processing artifact was excluded. In addition, as not to exhaust the tissue, small-size (<1 cm) cases were excluded. For an additional 31 cases, whole sections from surgical specimen blocks were used.

Immunohistochemistry

All immunohistochemistry was performed as described previously.[21,22] The slides were reviewed by two pathologists (APS and PA) to confirm the interpretation of results. Immunohistochemistry for ER (monoclonal, 1:1 dilution, Ventana) and PR (monoclonal, 1:60 dilution, Dako) were performed on all cases as part of a routine panel. Cases demonstrating weak, moderate or strong nuclear labeling for ER or PR in $>1\%$ of cells were considered ER positive or PR positive, respectively. Immunohistochemistry for HER-2 was performed on all cases as part of a routine panel using the Dako Herceptest kit and following the manufacturer's guidelines. Cases were scored using established criteria as 0 or 1+ (negative), 2+ (equivocal) and 3+ (positive). FISH analysis for HER-2 amplification was performed on all cases with 2+ (equivocal) immunohistochemistry results using the Path Vysion kit (Vysis-Abbott Molecular). Cases with either a 3+ (strong positive) immunohistochemistry score or a HER-2 FISH amplification ratio >4 were considered HER-2-positive. Cases with low-level amplification (ratios 2.2–4.0) were excluded from this study because of their uncertain clinical significance. To determine the basal subtype, CK 5/6 (monoclonal, Dako) and EGFR (monoclonal, 1:50 dilution, Zymed) immunohistochemistry was performed on whole sections from a subset of cases (29

of 37), which were negative for ER, PR and HER-2. For CK 5/6, cases were scored on the basis of the percentage of positive cells: 1+ (1–25%), 2+ (26–50%), 3+ (51–75%) and 4+ (76–100%). Cases with membranous or cytoplasmic labeling in >25% of neoplastic cells were considered positive. For EGFR, cases were scored on the basis of the percentage of positive cells: 1+ (1–25%), 2+ (26–50%), 3+ (51–75%) and 4+ (76–100%). Any strong membranous staining for EGFR was considered positive, generally labeling 10–50% of neoplastic cells. p53 (monoclonal, Ventana) and Ki-67 (monoclonal, Ventana) immunohistochemistry were also performed and only nuclear labeling was scored. For p53, labeling of >30% of nuclei was considered aberrant overexpression, and labeling of <30% of nuclei was considered negative for aberrant overexpression; 30% labeling of nuclei was considered equivocal. For Ki-67, the labeling of ≥20% of nuclei was considered high and the labeling of <20% of nuclei was considered low.

Telomere FISH

Telomere lengths were assessed by fluorescence staining for telomeric DNA as described previously.[6,23] In brief, deparaffinized slides were hydrated through a graded ethanol series, placed in deionized water, followed by deionized water plus 0.1% Tween-20. Slides were then placed in citrate buffer (catalog no. H-3300; Vector Laboratories), steamed for 14 min (Black and Decker Handy Steamer Plus; Black and Decker), removed and allowed to cool at room temperature for 5 min. Slides were then placed in PBS with Tween (catalog no. P-3563; Sigma Chemical Co.) for 5 min. Slides were thoroughly rinsed with deionized water, followed by 95% ethanol for 5 min and then air dried. In all 25 μl of Cy3-labeled telomere-specific peptide nucleic acid (0.3 μg/ml peptide nucleic acid in 70% formamide, 10 mmol/l Tris, pH 7.5, 0.5% B/M Blocking reagent (catalog no. 1814-320; Boehringer-Mannheim)) was applied to the sample, which was then coverslipped, and denaturation was performed by incubation for 4 min at 83°C. Slides were then transferred to a dark, closed container for hybridization at room temperature for 2 h. Coverslips were then carefully removed and slides were washed twice in peptide nucleic acid wash solution (70% formamide, 10 mmol/l Tris, pH 7.5, 0.1% albumin (from 30% albumin solution, catalog no. A-7284; Sigma Chemical Co.)) for 15 min each, and then rinsed in PBS with Tween and thoroughly rinsed in deionized water. Slides were drained and counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (500 ng/ml in deionized water, Sigma Chemical Co. catalog no. D-8417) for 5 min at room temperature, mounted with Prolong anti-fade mounting medium (catalog no. P-7481; Molecular Probes) and then imaged. The peptide nucleic acid probe complementary to the mammalian telomere repeat sequence was obtained from Applied Biosystems, and has the sequence (N terminus to C terminus) CCCTAACCCTAACCCTAA with an N-terminal covalently linked Cy3 fluorescent dye. As a positive control for hybridization efficiency, an FITC-labeled peptide nucleic acid probe having the sequence ATTCGTTGGAAACGGGA with specificity for human centromeric DNA repeats (CENP-B-binding sequence) was also included in the hybridization solution.[24]

Microscopy and Telomere Length Assessment

Slides were imaged using a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, Canada). Fluorescence excitation/emission filters were as follows: Cy3 excitation, 546 nm/10 nm BP; emission, 578 nm LP (Carl Zeiss Inc.); DAPI excitation, 330 nm; emission, 400 nm using an XF02 fluorescence set (Omega Optical, Brattleboro, VT, USA); Alexa Fluor 488 excitation, 475 nm; emission, 535 nm by a combination of 475RDF40 and 535RDF45 filters (Omega Optical). Gray scale images of representative regions were captured for presentation using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged. Integration times typically ranged from 500 to 800 ms for Cy3 (telomere) and FITC (centromere) signal capture, and 50–100 ms for the DAPI counterstain. Telomere lengths were qualitatively scored by direct visual assessment of stained slides, comparing telomere signals from tumor cells with telomere signals from benign cells (stromal cells and/or myoepithelial cells) from the same case. In all cases, signals from benign cells were considered 3+. Telomeres in tumor cells of different cases ranged from short (0+, 1+, or 2+) to normal (3+) to long (4+, 5+).

Statistical Analysis

For all analyses, normal and long telomere groups were combined and compared with the short telomere group. Results were compared using two-sided Fisher's exact tests. *P*-values <0.05 were considered to be significant. SAS 9.2 and JMP® statistical packages (SAS Institute, Cary, NC, USA) were used for all analyses.

Results

Clinical and Pathological Characteristics

Table 1 lists the clinical and pathological characteristics of the study population. Of the 103 subjects included in this study, the mean age at diagnosis for all patients was 56 years (range: 30–94 years). Patients were predominantly Caucasian (57%) or

Table 1 Clinical and pathological characteristics for all patients and stratified by telomere length

Characteristics	*All subjects* (n = *103*)	*Telomere length*[a]	
		Short (n = *88*)	*Normal/long* (n = *15*)
Age at diagnosis (years)			
Mean (range)	56 (30–94)	55 (30–92)	61 (34–94)
Weight (kg)[b]			
Mean (range)	79.1 (44.0–163.5)	79.9 (46.8–163.5)	73.8 (44.0–97.7)
Ethnicity			
Caucasian	59	46	13
African American	32	30	2
Other	12	12	0
Parity status			
Yes	82	73	9
No	20	14	6
Missing	1	1	0
Menopausal status			
Pre-menopausal	41	35	6
Post-menopausal	60	52	8
Missing/uncertain	2	1	1
Elston grade			
1	4	1	3
2	24	20	4
3	75	67	8
TNM stage			
I	22	22	0
II	61	52	9
III	19	13	6
IV	1	1	0

[a]There are no statistically significant differences in any of the clinical or pathological characteristics between the telomere length groups.
[b]In all, 18 women are missing weight data.

African American (31%). Elston grading of tumors showed that 4% were grade I, 23% were grade II and 73% were grade III. According to the established criteria of the American Joint Committee on Cancer (AJCC 2007), 21% were stage I, 59% were stage II, 18% were stage III and 1% were stage IV. Interestingly, the short telomere length group tended to be younger and contained a greater proportion of African-American women when compared with the normal/long group; however, these differences did not reach statistical significance.

Telomere-FISH in Breast Tumors

Telomere length was qualitatively scored and grouped into short ($n = 88$), normal ($n = 13$) or long ($n = 2$) categories. Figure 1a shows a representative example of breast cancer with short telomeres as indicated by diminished telomere signals in cancer cells when compared with either stromal cells or myoepithelial cells in an adjacent terminal ductal lobular unit. As shown in Figure 1b, cancer cells from this breast cancer show comparable telomere intensities to that observed in the surrounding benign stroma. Figure 1c, shows an example of a breast tumor with increased telomere signals in cancer cells when compared with the surrounding benign stromal cells. Table 1 shows the clinical and pathological characteristics for the subset of patients with short telomeres ($n = 88$) and the subset of patients with either normal or long telomeres ($n = 15$).

Hormone Receptor Expression Characteristics

Table 2 shows tumor characteristics including ER status, PR status, HER-2, p53 and Ki-67 stratified by telomere length. ER-negative ($P = 0.022$), PR-negative ($P = 0.008$) and HER-2-positive ($P = 0.023$) tumors were significantly more likely to have a greater fraction of short telomeres when compared with the normal/long telomere group. In addition, there was a significant increase in the proportion of p53-positive tumors in the short telomere category ($P = 0.022$). Shorter telomere length was also more prevalent in tumors with an increased Ki-67 index ($\geq 20\%$ of cells positive), but it did not reach statistical significance.

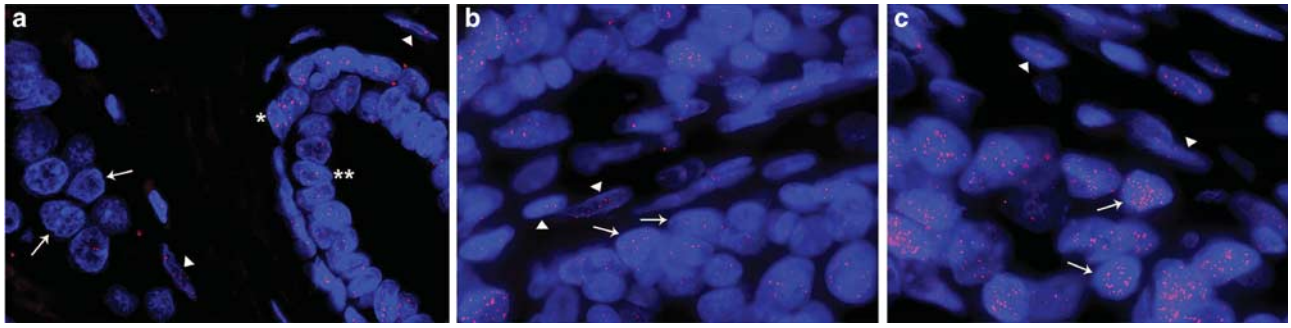

Figure 1 Telomere length analysis by FISH in breast adenocarcinomas. Three representative examples of cases showing short, normal or long telomere lengths in cancer cells are shown. (**a**) This case shows strikingly diminished telomere signals in tumor cells as compared to the surrounding benign stroma and in an adjacent terminal ductal lobular unit with myoepithelial (*) and luminal (**) cells. (**b**) This case displays comparable telomere intensities in tumor cells with those observed in the surrounding benign stroma. (**c**) In this case, cancer cells show extremely bright telomere signals in cancer cells when compared with the surrounding benign stroma. In all the images, the DNA is stained with DAPI (blue) and telomere DNA is stained with the Cy3-labeled telomere-specific peptide nucleic acid probe (red). It is noteworthy that the centromere DNA, stained with the FITC-labeled centromere-specific peptide nucleic acid probe, has been omitted from the image to emphasize the differences in telomere lengths. In all panels, the arrows point to cancer cells and the arrowheads point to benign stromal cells. Original magnification, $\times 400$.

Table 2 Association between ER, PR, HER-2, p53 and Ki-67 expression characteristics stratified by telomere length

Characteristics	*Telomere length*		P-*value*
	Short	*Normal/long*	
ER status			
Positive	34	11	
Negative	54	4	0.022
PR status			
Positive	30	11	
Negative	58	4	0.008
HER-2			
Positive	53	4	
Negative	35	11	0.023
p53[a]			
Positive	40	2	
Negative	46	13	0.022
Ki-67[b]			
High ($\geq$20%)	70	8	
Low ($<$20%)	17	6	0.082

[a]Two patients with equivocal p53 staining were not included in the analysis.
[b]Two patients are missing Ki-67 data.

Subtype Characterization and Telomere Length Assessment

Cases were categorized into one of four groups; luminal A, luminal B, HER-2-positive or triple-negative. In all, 18 cases were ER/PR positive/HER-2 negative, and were considered luminal A cases. There were 28 luminal B cases that were HER-2-positive and ER and/or PR positive. A total of 20 tumors were HER-2-positive and negative for ER and PR, respectively, and were considered HER-2-positive cases. In addition, there were 37 triple-negative carcinoma cases which were negative for ER, PR and HER-2. For 29 of the triple-negative cases, immunohistochemical data on CK 5/6 and EGFR expression were available, allowing assessment of the basal phenotype. In all, 18 of these cases showed staining for CK 5/6 and/or EGFR (basal) and 11 cases were CK 5/6 and EGFR negative (non-basal). Within this triple-negative group, there was no difference in the proportion of cases with short telomeres between the subset of cases determined to be basal (94%) and non-basal (91%) phenotypes. As shown in Figure 2, luminal B cancers (93%), HER-2 cancers (90%) and triple-negative cancers (95%) had an increased proportion of cases with short telomeres when compared with luminal A cancers (50%); these were all statistically significant differences ($P=0.002$, $P=0.011$ and $P=0.0003$, respectively).

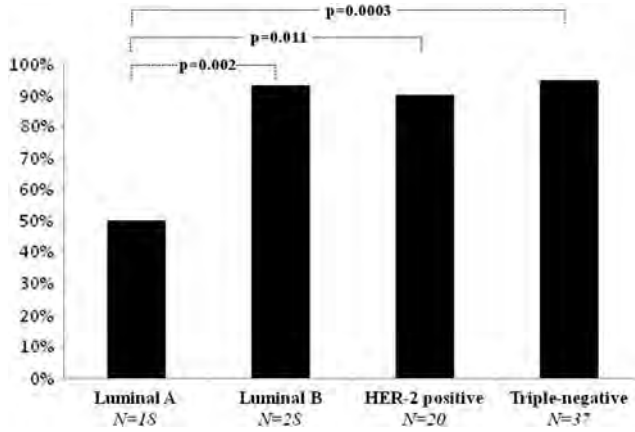

Figure 2 Proportion of cases with short telomeres among different subtypes of breast cancer. *P*-values were determined using two-sided Fisher's exact tests.

Discussion

The first principal conclusion emerging from this study is the observation that telomere shortening is

associated with other established breast cancer prognostic factors. It is well established that breast cancer patients with tumors that are ER and/or PR negative have an increased risk of mortality.[25–27] We observed an increased proportion of tumors in the short telomere group that were negative for ER and PR. Although temporality cannot be determined, these results suggest that telomere shortening may contribute to the selection of cells capable of growing in the absence of hormone receptors. Another molecular marker associated with poor breast cancer prognosis is the presence of mutations in the p53 gene, predominantly missense mutations leading to conformational alterations of the protein and accumulation in the tumor cell nuclei.[28–30] We observed an increased proportion of tumors in the short telomere group that were p53 positive, suggesting that less-aggressive tumors are characterized by normal length telomeres and no mutations in the *p53* gene. Conversely, short, dysfunctional telomeres may provide a strong selective pressure for abrogation of the p53 pathway. Normally, critical telomere shortening leads to p53-dependent tumor-suppressive cellular responses, such as cellular senescence and apoptosis.[2] However, if this checkpoint is abrogated and the telomeres are partially stabilized by upregulation of telomerase, then further proliferation occurs and genomic instability may accumulate.[31,32]

The second principal conclusion emerging from this study is that telomere shortening occurs in the vast majority of luminal B, HER-2 and triple-negative tumors, but in a smaller fraction of luminal A type tumors. A recent large patient cohort demonstrated that luminal A tumors are associated with a lower risk of local or regional recurrence when compared with other molecular subtypes.[18] It has long been recognized that breast adenocarcinomas are characterized by genomic instability. The molecular mechanisms leading to genomic instability are not fully elucidated; however, one potential initiator of genomic instability is telomere dysfunction due to critical telomere shortening.[4,33] Thus, telomere shortening may be reflective of the degree of underlying genomic instability, a feature shared by higher-grade HER-2 and triple-negative carcinomas.[34] Another possibility is that telomere shortening follows tumor progression as evidenced by the association with higher stage and histological grade.[8,10,11] It is noteworthy that recent data have demonstrated that not only can short telomeres lead to telomere dysfunction but abnormally long telomeres may also do so.[35,36] In this context, we previously demonstrated the presence of alternative lengthening of telomeres, a recombination-based mechanism that lengthens telomeres, in three cases of breast carcinoma.[21] Interestingly, all three cases were also HER-2-positive, suggesting a possible common underlying mechanism. The significance of long telomeres in cancers lacking evidence of the alternative lengthening of telomeres phenotype is currently not clear and would need to be addressed in future studies.

This study is the first assessment of telomere lengths in breast cancer subtypes. In addition, we analyzed telomere lengths directly, at the single cell level, within breast cancer tissues using a FISH assay. Previous studies have measured telomeres using bulk assays such as Southern blot and terminal restriction fragment analysis,[8] quantitative PCR[10] or with a chemiluminescent-based slot blot assay that measures telomere DNA content, a proxy of telomere length.[9,11,12] These types of measurements do not allow for single cell resolution. Study limitations include the absence of small tumors (<1 cm), thus selecting for higher stage and histological grade cases, and the limited demographic and lifestyle information, as this was obtained retrospectively. In addition, the lack of complete data on follow-up time, because of the fact that part, but not all, of the patients' treatment occurred at our Institution, prevented us from determining an association between telomere length and outcome.

In conclusion, we have demonstrated that telomere lengths vary among different subtypes of breast cancer in a manner consistent with their aggressiveness. Prospective studies are required to further evaluate the usefulness of telomere length as a prognostic and/or predictive marker of disease progression and treatment response within the various subtypes and determine temporality.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Short Communication

Prevalence of the Alternative Lengthening of Telomeres Telomere Maintenance Mechanism in Human Cancer Subtypes

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Approximately 10% to 15% of human cancers lack detectable telomerase activity, and a subset of these maintain telomere lengths by the telomerase-independent telomere maintenance mechanism termed alternative lengthening of telomeres (ALT). The ALT phenotype, relatively common in subtypes of sarcomas and astrocytomas, has rarely been reported in epithelial malignancies. However, the prevalence of ALT has not been thoroughly assessed across all cancer types. We therefore comprehensively surveyed the ALT phenotype in a broad range of human cancers. In total, two independent sets comprising 6110 primary tumors from 94 different cancer subtypes, 541 benign neoplasms, and 264 normal tissue samples were assessed by combined telomere-specific fluorescence *in situ* hybridization and immunofluorescence labeling for PML protein. Overall, ALT was observed in 3.73% (228/6110) of all tumor specimens, but was not observed in benign neoplasms or normal tissues. This is the first report of ALT in carcinomas arising from the bladder, cervix, endometrium, esophagus, gallbladder, kidney, liver, and lung. Additionally, this is the first report of ALT in medulloblastomas, oligodendrogliomas, meningiomas, schwannomas, and pediatric glioblastoma multiformes. Previous studies have shown associations between ALT status and prognosis in some tumor types; thus, further studies are warranted to assess the potential prognostic significance and unique biology of ALT-positive tumors. These findings may have therapeutic consequences, because ALT-positive cancers are predicted to be resistant to anti-telomerase therapies. ***(Am J Pathol 2011, 179:1608–1615; DOI: 10.1016/j.ajpath.2011.06.018)***

Telomeres are the nucleoprotein complexes located at the extreme ends of eukaryotic chromosomes; they consist of 5 to 10 kb of the repeating hexanucleotide DNA sequence TTAGGG.[1,2] The shelterin complex, a core set of six proteins integral for telomere function, associates with these repetitive DNA regions.[3,4] Telomeres function primarily to mask double-strand break DNA damage signals at chromosomal termini, inhibit terminal exonucleolytic degradation, and prevent chromosomal fusions.[5,6] In normal somatic cells, telomeres shorten with each cell division, and significant telomere shortening leads to p53-dependent senescence or apoptosis.[7] As a result, there is a limited number of population doublings that a

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somatic cell lineage may undergo, at which point further proliferative expansion is blocked. During malignant transformation, these cell cycle checkpoints are abrogated (eg, through mutations in tumor suppressor proteins). If cellular proliferation continues unchecked, then genomic instability may ensue via chromosomal breakage-fusion-bridge cycles caused by eroded, dysfunctional telomeres.[8] In 85% to 90% of human cancers, telomere dysfunction is attenuated and telomere length appears to be maintained, or increased, through up-regulation of the enzyme telomerase, a reverse transcriptase with the ability to synthesize new telomere DNA using an internal RNA template.[9] However, telomere loss may also be compensated in some cancers by the telomerase-independent telomere maintenance mechanism termed alternative lengthening of telomeres (ALT), which is thought to be dependent on homologous recombination.[10]

The ALT phenotype is identified at the cellular level by the presence of ALT-associated promyelocytic leukemia (PML) protein nuclear bodies (APBs) that contain large amounts of telomeric DNA, in addition to PML protein and other proteins involved in telomere binding, DNA replication, and recombination.[11,12] ALT-positive cells are characterized by striking telomere length heterogeneity, as well as increased chromosomal instability. APBs are cancer-specific and, in fixed tissues, can be visualized by combined telomere-specific fluorescence *in situ* hybridization (FISH) and immunofluorescence labeling for PML protein.[13,14] This method has been extensively validated and allows for straightforward identification of ALT-positive cancers in fixed human tissue specimens.[15]

The ALT phenotype is common among certain sarcomas (eg, osteosarcomas and liposarcomas), as well as in subsets of central nervous system tumors, including astrocytomas[16]; however, the prevalence of ALT varies widely among these different tumor types. Our laboratory recently reported the presence of ALT in a small subset of breast carcinomas,[17] but the ALT phenotype has rarely been reported in other epithelial malignancies.[16]

We have comprehensively surveyed the ALT phenotype in two independent sets of fixed specimens, comprising a total of 6110 primary tumors from a broad range of human cancer subtypes. Overall, the prevalence of the ALT phenotype is 3.73%; however, the prevalence varies drastically between different subtypes. Here, we describe the results of this extensive survey, including the novel finding of the ALT phenotype in carcinomas arising from the bladder, cervix, endometrium, esophagus, gallbladder, kidney, liver, and lung. In addition, this is the first report of the ALT phenotype in several tumor types of nonepithelial origin, including medulloblastomas, pediatric glioblastomas multiformes (GBMs), oligodendrogliomas, meningiomas, and schwannomas.

Materials and Methods

Sources of Tissue

Two independent sets of primary tumor tissues were used in the present study. Set 1 consisted of 4001 tumors from 68 different cancer subtypes. The vast majority of these cases were resected and processed at the Johns Hopkins Hospital and were arrayed in tissue microarray (TMA) format. This set consisted of 165 TMAs containing multiple cores of each tumor specimen and, in most instances, adjacent normal tissue. In addition to these TMAs from our institution, seven TMAs containing 195 cases (three cores per case from cancer and one core from matched normal intestinal mucosa) of primary small intestinal adenocarcinoma from 20 institutions of the Korean Small Intestinal Cancer Study Group were included. Moreover, 56 invasive breast carcinoma tissue sections from the Johns Hopkins Hospital and 29 neuroblastic tumor tissue sections from the University of Texas Southwestern Medical Center were also obtained. To validate and expand on the findings in this first set, a second set of multitumor arrays was obtained (set 2).[18] TMAs in set 2 contained 2109 primary tumors from 61 cancer subtypes. In this set of tumors, each case was represented on the array by a single tissue core. In addition to the malignant tumors, 541 benign neoplasms (see Supplemental Table S1 at *http://ajp.amjpathol.org*) and 264 normal tissue samples (see Supplemental Table S2 at *http://ajp.amjpathol.org*) were also obtained. The study was approved by the Johns Hopkins University School of Medicine institutional review board.

Telomere-Specific Immunostaining FISH

Combined telomere-specific FISH and immunofluorescence labeling of PML protein was performed as described previously.[13,14] Briefly, deparaffinized slides were hydrated, steamed for 25 minutes in citrate buffer (Vector Laboratories, Burlingame, CA), dehydrated, and hybridized with a Cy3-labeled peptide nucleic acid (PNA) probe complementary to the mammalian telomere repeat sequence [(N-terminus to C-terminus) 5′-CCCTAACCCTAACCCTAA-3′]. As a positive control for hybridization efficiency, an Invitrogen (Carlsbad, CA) Alexa Fluor 610-labeled PNA probe having specificity for human centromeric DNA repeats (5′-ATTCGTTGGAAACGGGA-3′, CENP-B binding sequence) was included in the hybridization solution.[19] After posthybridization washes, an anti-PML antibody (1:100 dilution; catalog no. PG-M3; Dako, Carpinteria, CA) was incubated for 45 minutes at room temperature, followed by incubation of anti-mouse Alexa Fluor 488 fluorescent secondary antibody (catalog no. A-11001; Molecular Probes, Eugene, OR) and counterstaining with DAPI. Slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions, Mississauga, ON, Canada) and appropriate fluorescence excitation/emission filters. Grayscale images were captured using Nikon NIS-Elements software version 2.30 and an attached Photometrics (Tucson, AZ) CoolSNAP EZ digital camera, pseudo-colored, and merged.

ALT Assessment

All cases were assessed for the presence of the ALT phenotype. ALT-positive cases were identified by large,

very bright intranuclear foci of telomere FISH signals marking ALT-associated telomeric foci throughout the tumor cells. Although telomere FISH signals from these individual bright foci often colocalized with PML protein, heterogeneity in this trait was observed, even within the same tumor. Given several instances in the literature of ALT-positive cell lines lacking telomere/PML colocalization,[20–22] colocalization was not considered an absolute requirement for classifying a case as ALT-positive. Thus, cases were classified as ALT-positive if they met the following criteria: first, the presence of ultra-bright intranuclear foci of telomere FISH signals (ALT-associated telomeric foci), with integrated total signal intensities for individual foci being >10-fold that of the per cell mean integrated signal intensities for all telomeric signals in individual benign stromal cells within the same case; second, ≥1% of tumor cells displaying these ALT-associated telomeric foci. Cases lacking ALT-associated telomeric foci in which at least 500 cells were assessed were considered ALT-negative. Areas exhibiting necrosis were excluded from consideration.

Statistical Analysis

When appropriate, different tumor subtypes were compared with a two-sided Fisher's exact test using SAS version 9.2 statistical packages (SAS Institute, Cary, NC). *P* values of <0.05 were considered to be significant.

Results

Determination of the ALT Telomere Maintenance Mechanism in Human Cancer Subtypes

We identified the presence of the ALT phenotype by using telomere-specific FISH to visualize telomeric DNA in interphase nuclei of fixed tissue specimens. ALT-positive tumors are readily distinguishable by large ultra-bright telomere FISH signals, which are a nearly universal feature of ALT-positive cell populations.[23] In Figure 1, we present for comparison an ALT-negative invasive urothelial carcinoma case (Figure 1A) and an ALT-positive invasive urothelial carcinoma case (Figure 1B). The ALT-negative case displays robust telomere signals in the tumor cells and adjacent stromal cells; in the ALT-positive case, distinctive large, very bright intranuclear foci of telomere FISH signals mark ALT-associated telomeric foci throughout the tumor cells. Other representative ALT-positive cases shown include a renal sarcomatoid carcinoma (Figure 1C) and an anaplastic medulloblastoma (Figure 1D), neither of which has been previously identified as using the ALT pathway. In ALT-positive tumors, the percentage of cells containing ALT-associated telomeric foci varied by tumor type, ranging from 1% to >95% of tumor cells. This trait also varied among different tumors from the same cancer subtype. Finally, in Figure 1 we present two additional ALT-positive cases, an oligodendroglioma (Figure 1E) and an angiosarcoma (Figure 1F). In both of these cases, PML protein colocalizes to most of the ALT-associated telomere foci. The inset for each case highlights a typical APB, displaying a targetoid appearance of telomere DNA signal with a peripheral rim of PML protein.

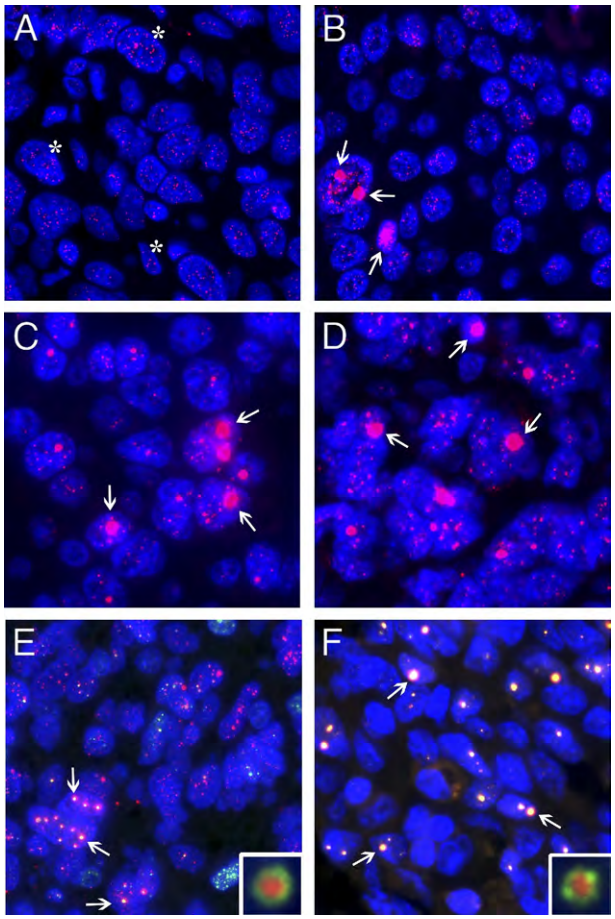

Figure 1. Representative examples of ALT-negative and ALT-positive tumors. **A** and **B:** Representative invasive urothelial carcinomas. In the ALT-negative case (**A**), robust telomere signals are present in tumor cells and adjacent stromal cells (**asterisks**). In the ALT-positive case (**B**), distinctive large, very bright intranuclear foci of telomere FISH signals mark ALT-associated telomeric foci throughout the tumor cells (**arrows**). Note the marked heterogeneity in the telomere signals, where visible, in the cancer cells. **C–F:** Representative ALT-positive cases of renal sarcomatoid carcinoma (**C**), anaplastic medulloblastoma (**D**), oligodendroglioma (**E**), and angiosarcoma (**F**). In all images (**A–F**), the DNA is stained with DAPI (blue) and telomere DNA is stained with the Cy3-labeled telomere-specific PNA probe (red). Two cases (**E** and **F**) are shown with costaining of PML protein (green), to demonstrate colocalization to most of the ALT-associated telomeric foci (**arrows**). The **inset** for each case highlights a typical APB that contains a targetoid appearance of telomere signal with a peripheral rim of PML protein. Original magnification: ×400 (**A–F**); ×1000 (**inset**).

Prevalence of the ALT Telomere Maintenance Mechanism in Human Cancer Subtypes

To determine the prevalence of the ALT phenotype in human cancers, we assessed two independent sets of malignant tissues comprising 6110 primary tumors from 94 different cancer subtypes. Set 1 consisted of 4001 specimens encompassing a broad range of malignant tumors, including tumors arising from the adrenal gland, biliary tract, breast, central nervous system, colon, esophagus, gallbladder, kidney, liver, lung, ovary, pancreas, prostate, salivary gland, skin, small intestine, soft

tissue, stomach, urinary bladder, and uterus (Table 1). A total of 141 tumors were identified as ALT-positive in set 1, yielding a prevalence of 3.52%. To confirm and expand on these findings, we further assessed the ALT phenotype in a second set of multitumor TMAs (set 2), which had previously been used to validate other molecular markers.[18] This set of tumors consisted of 2109 primary tumor specimens from 61 different cancer subtypes. Set 2 included types similar to the first set, but also included hematopoietic and neuroendocrine neoplasms, as well as tumors arising from the oral cavity, pleura, tendon sheath, testis, and thyroid (Table 1). A total of 87 tumors were identified as ALT-positive in set 2, representing a prevalence of 4.13%. With cases from both sets combined, a total of 228 ALT-positive tumors were identified, representing an overall prevalence of the ALT phenotype in human cancers of 3.73% (Table 1).

First Description of the ALT Telomere Maintenance Mechanism in Numerous Cancer Subtypes

Although we recently described the presence of the ALT phenotype in a small subset of breast carcinomas,[17] the ALT phenotype has rarely been reported in other epithelial malignancies.[16] Here, we report the presence of the ALT phenotype in numerous epithelial malignancies. The ALT phenotype was present in 8/121 (7%) cases of hepatocellular carcinoma, 3/41 (7%) cases of serous endometrial carcinoma, 3/152 (2%) cases of squamous cervical carcinoma, 1/60 (2%) case of gallbladder adenocarcinoma, and 1/106 (1%) case of esophageal adenocarcinoma. In renal carcinoma, the ALT phenotype was observed in 4/47 (9%) cases of chromophobe carcinoma, 2/27 (7%) cases of sarcomatoid carcinoma, 1/117 (1%) case of clear cell carcinoma, and 1/86 (1%) case of papillary carcinoma. In urinary bladder carcinomas, we observed the presence of ALT in 3/13 (7%) cases of small cell bladder carcinoma and 2/150 (1%) cases of invasive urothelial carcinoma. Although ALT was not observed in most lung carcinoma subtypes, we did observe a single case each of large cell [1/35 (3%)] and small cell [1/63 (2%)] carcinomas that exhibited the ALT phenotype.

In addition to the novel findings in epithelial malignancies, we present here the first report of ALT in several tumor types of nonepithelial origin, including medulloblastomas, pediatric GBMs, oligodendrogliomas, meningiomas, and schwannomas. In medulloblastomas, the prevalence of ALT-positive tumors varied across subtypes: 18% in anaplastic medulloblastomas and 3% in nonanaplastic medulloblastomas. The prevalence of the ALT phenotype in adult GBM cases was 11%. We also assessed 32 cases of pediatric GBM and observed a statistically significant increase in the prevalence of the ALT phenotype in the pediatric cases (44%), compared with the adult cases ($P = 0.0002$). In other central nervous system tumors, the prevalence of ALT was 20% in oligodendrogliomas, 2% in meningiomas, and 2% in schwannomas.

The ALT Telomere Maintenance Mechanism Is Not Observed in Numerous Cancer Subtypes

There appear to be several cancer subtypes that rarely, if ever, use the ALT telomere maintenance mechanism. In particular, we did not observe the ALT phenotype in adenocarcinomas arising from the prostate ($N = 1152$), pancreas ($N = 448$), small intestine ($N = 215$), stomach ($N = 155$), or colon ($N = 126$). Although the numbers of cases were smaller, we also did not observe the ALT phenotype in cholangiocarcinomas, laryngeal squamous cell carcinomas, oral squamous cell carcinomas, salivary gland carcinomas, follicular and papillary thyroid carcinomas, giant cell tumors of the tendon sheath, or hematopoietic neoplasms. Although malignancies arising from certain organs (eg, prostate cancer) rarely, if ever, develop ALT, there are malignancies from other organ sites that are capable of developing the ALT phenotype, but apparently only in particular cancer subtypes. Notably, in lung carcinoma, the ALT phenotype was observed only in a small subset of carcinomas originating from neuroendocrine cells; it was not observed in any other subtype. Other specific subtypes in which we did not observe the ALT phenotype include ovarian serous carcinoma, endometrioid carcinoma of the endometrium, seminoma, and basal cell and squamous cell carcinoma of the skin. Although the ALT phenotype is highly prevalent in certain types of sarcomas, we did not find evidence of the ALT phenotype in gastrointestinal stromal tumors, Kaposi's sarcomas, or Ewing's sarcomas/primitive neuroectodermal tumors.

The ALT Telomere Maintenance Mechanism Is Not Observed in Normal Tissue Samples or Benign Neoplasms

Next, we assessed a set of 264 normal tissues encompassing a wide range of tissue types. The ALT phenotype was not observed in these non-neoplastic tissue samples (see Supplemental Table S1 at *http://ajp.amjpathol.org*). In accord with this observation, we did not observe the ALT phenotype in non-neoplastic tissue entrapped in or adjacent to any of the tumors assessed. We also assessed a set of 541 benign neoplasms arising from a range of different tissues. The ALT phenotype was not observed in these benign neoplasms (see Supplemental Table S1 at *http://ajp.amjpathol.org*). Although we did not specifically assess intraepithelial neoplasms, we did observe the ALT phenotype in two individual cases: a melanoma *in situ* and a case of cervical intraepithelial neoplasia (grade 3). For representative images demonstrating the presence of ALT-associated telomeric foci in these cases (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Discussion

In the present study, we comprehensively surveyed the ALT telomere maintenance mechanism in a broad range of human cancer subtypes. We used telomere-specific FISH and immunofluorescence labeling for PML protein

Table 1. Prevalence of the ALT Phenotype in Human Cancer Subtypes

Location/Tumor type	ALT$^+$, set 1		ALT$^+$, set 2		ALT$^+$, overall	
	n/N	%	*n/N*	%	*n/N*	%
Adrenal gland/peripheral nervous system						
Pheochromocytoma	1/39	3	1/28	4	2/67	3
Neuroblastoma	2/22	9	—*	—	2/22	9
Ganglioneuroblastoma	1/7	14	—	—	1/7	14
Biliary						
Cholangiocarcinoma, extrahepatic	0/23	0	—	—	0/23	0
Cholangiocarcinoma, intrahepatic	0/10	0	—	—	0/10	0
Breast						
Ductal carcinoma†	5/217	2	0/34	0	5/251	2
Ductal carcinoma with lobular features	0/20	0	—	—	0/20	0
Lobular carcinoma	1/14	7	0/13	0	1/27	4
Mucinous carcinoma	—	—	0/15	0	0/15	0
Tubular carcinoma	—	—	0/9	0	0/9	0
Medullary carcinoma	0/1	0	1/54	2	1/55	2
Central nervous system						
Pilocytic astrocytoma (grade 1)	2/55	4	0/3	0	2/58	3
Diffuse astrocytoma (grade 2)	14/19	74	3/8	38	17/27	63
Anaplastic astrocytoma (grade 3)	17/19	89	2/11	18	19/30	63
Glioblastoma multiforme (grade 4; adult)	9/65	14	3/40	8	12/105	11
Glioblastoma multiforme (grade 4; pediatric)	14/32	44	—	—	14/32	44
Oligodendroglioma	6/20	30	2/20	10	8/40	20
Medulloblastoma, anaplastic	3/17	18	—	—	3/17	18
Medulloblastoma, nonanaplastic	1/38	3	—	—	1/38	3
Other embryonal tumors	1/10	10	—	—	1/10	10
Meningioma	—	—	1/46	2	1/46	2
Schwannoma	—	—	1/44	2	1/44	2
Colon						
Adenocarcinoma	0/77	0	0/49	0	0/126	0
Esophagus						
Adenocarcinoma	0/97	0	1/9	11	1/106	1
Squamous cell carcinoma	—	—	0/29	0	0/29	0
Small cell carcinoma	—	—	0/1	0	0/1	0
Gallbladder						
Adenocarcinoma	1/27	4	0/33	0	1/60	2
Hematopoietic neoplasms						
non-Hodgkin's lymphoma, other subtypes	—	—	0/54	0	0/54	0
non-Hodgkin's lymphoma, diffuse large B-cell	—	—	0/10	0	0/10	0
Hodgkin's lymphoma, nodular sclerosis	—	—	0/23	0	0/23	0
Hodgkin's lymphoma, mixed cellularity	—	—	0/17	0	0/17	0
Thymoma	—	—	0/37	0	0/37	0
Kidney						
Clear cell carcinoma	1/69	1	0/48	0	1/117	1
Papillary carcinoma	0/54	0	1/32	3	1/86	1
Chromophobe carcinoma	3/37	8	1/10	10	4/47	9
Sarcomatoid carcinoma	2/27	7	—	—	2/27	7
Larynx						
Squamous cell carcinoma	—	—	0/29	0	0/29	0
Liver						
Hepatocellular carcinoma	7/91	8	1/30	3	8/121	7
Lung						
Adenocarcinoma	0/64	0	0/89	0	0/153	0
Squamous cell carcinoma	0/55	0	0/45	0	0/100	0
Papillary carcinoma	0/45	0	—	—	0/45	0
Bronchioloalveolar carcinoma	0/40	0	—	—	0/40	0
Small cell carcinoma	0/16	0	1/47	2	1/63	2
Large cell carcinoma	0/10	0	1/25	4	1/35	3
Carcinoma, other subtypes	0/15	0	—	—	0/15	0
Carcinoid tumor	0/3	0	—	—	0/3	0
Neuroendocrine neoplasms						
Carcinoid tumor	—	—	2/32	6	2/32	6
Paraganglioma	—	—	1/8	13	1/8	13
Oral cavity						
Squamous cell carcinoma	—	—	0/41	0	0/41	0
Ovary						
Serous carcinoma	0/163	0	0/42	0	0/205	0
Clear cell carcinoma	2/56	4	—	—	2/56	4
Endometrioid carcinoma	0/32	0	1/40	3	1/72	1
Mucinous carcinoma	—	—	0/21	0	0/21	0

(*table continues*)

Table 1. *Continued*

Location/Tumor type	ALT$^+$, set 1		ALT$^+$, set 2		ALT$^+$, overall	
	n/N	%	*n/N*	%	*n/N*	%
Pancreas						
Adenocarcinoma	0/420	0	0/28	0	0/448	0
Pleura						
Malignant mesothelioma	—	—	1/28	4	1/28	4
Prostate						
Adenocarcinoma	0/1071	0	0/81	0	0/1152	0
Small cell carcinoma	0/24	0	—	—	0/24	0
Salivary gland						
Carcinoma	0/98	0	—	—	0/98	0
Skin						
Malignant melanoma	2/47	4	5/59	8	7/106	7
Basal cell carcinoma	—	—	0/57	0	0/57	0
Squamous cell carcinoma	—	—	0/56	0	0/56	0
Small intestine						
Adenocarcinoma	0/195	0	0/20	0	0/215	0
Soft tissues						
Gastrointestinal stromal tumor	0/34	0	—	—	0/34	0
Kaposi's sarcoma	0/33	0	0/22	0	0/55	0
Ewing's sarcoma/primitive neuroectodermal tumor	0/23	0	—	—	0/23	0
Undifferentiated pleomorphic sarcoma‡	15/22	68	18/30	60	33/52	63
Fibrosarcoma and variants	3/21	14	—	—	3/21	14
Leiomyosarcoma	11/13	85	20/46	43	31/59	53
Liposarcoma	3/10	30	6/28	21	9/38	24
Angiosarcoma	1/9	11	—	—	1/9	11
Epithelioid sarcoma	2/6	33	—	—	2/6	33
Clear cell sarcoma	0/5	0	—	—	0/5	0
Malignant peripheral nerve sheath tumor	0/4	0	—	—	0/4	0
Rhabdomyosarcoma	0/4	0	—	—	0/4	0
Chondrosarcoma	2/2	100	—	—	2/2	100
Neurofibroma	—	—	4/37	11	4/37	11
Stomach						
Adenocarcinoma	0/80	0	0/75	0	0/155	0
Tendon sheath						
Giant cell tumor	—	—	0/22	0	0/22	0
Testis						
Seminoma	—	—	0/48	0	0/48	0
Nonseminomatous germ cell tumor	—	—	7/46	15	7/46	15
Thyroid						
Follicular carcinoma	—	—	0/52	0	0/52	0
Papillary carcinoma	—	—	0/30	0	0/30	0
Urinary bladder						
Invasive urothelial carcinoma	2/75	3	0/75	0	2/150	1
Non-invasive urothelial carcinoma	—	—	0/38	0	0/38	0
Small cell carcinoma	3/13	23	—	—	3/13	23
Non-invasive papillary urothelial carcinoma	0/5	0	—	—	0/5	0
Squamous carcinoma	0/2	0	—	—	0/2	0
Sarcomatoid carcinoma	0/1	0	—	—	0/1	0
Uterus						
Cervix, squamous carcinoma	3/127	2	0/25	0	3/152	2
Cervix, adenocarcinoma	0/19	0	—	—	0/19	0
Endometrium, endometrioid carcinoma	0/16	0	0/48	0	0/64	0
Endometrium, serous carcinoma	1/9	11	2/32	6	3/41	7
Endometrium, mixed mesodermal tumor	0/4	0	—	—	0/4	0
Endometrium, clear cell carcinoma	0/3	0	—	—	0/3	0

*Subtype not included in this set.
†Includes data from samples previously published.[17]
‡Includes cases classified as malignant fibrous histiocytoma.

to assess the ALT status in fixed tissue specimens of 6110 primary tumors from 94 different cancer subtypes. Across all cancer subtypes, the prevalence of the ALT phenotype was 3.73%; however, the prevalence varied widely between different cancer subtypes. The results obtained in sets 1 and 2 are similar, except in leiomyosarcomas, for which there was a statistically significant difference in the prevalence between the two independent sets (85% versus 43%; $P = 0.012$).

Through this intensive characterization of the ALT phenotype in human cancer, we describe for the first time ALT-positive carcinomas arising from the bladder, cervix, endometrium, esophagus, gallbladder, kidney, liver, and lung. Although in some of these epithelial malignancies

we observed only single ALT-positive cases, other carcinoma subtypes displayed considerable frequencies of ALT-positivity. For example, 7% of hepatocellular carcinomas were ALT-positive; within carcinomas of the kidney, 9% of chromophobe carcinomas and 7% of sarcomatoid carcinomas were ALT-positive. Of note, in some tissues, it appears that the ALT phenotype is more prevalent in tumors arising from neuroendocrine cells. For example, the prevalence of ALT in small cell bladder cases was 23%, compared with only 1% in invasive urothelial carcinoma. Similarly, individual cases of small cell and large cell lung carcinomas were observed; ALT was not present in any other lung carcinoma subtype.

In contrast to a previous study by Au et al,[24] wherein ALT was not observed in malignant pleural mesotheliomas ($N = 43$), in the present study we observed a single ALT-positive case of this cancer ($N = 28$). Two additional cancer types that had previously been determined to contain a small subset of ALT-positive tumors were confirmed and extended. Previously, Bryan et al[10] observed abnormally long telomeres (by Southern blotting) in 1/9 malignant melanomas and 2/15 ovarian carcinomas. In the present study, we found a prevalence of 7% in malignant melanomas, 4% in ovarian clear cell carcinomas, and 1% in endometrioid carcinomas of the ovary.

This is the first report of the ALT telomere maintenance mechanism in pediatric GBMs, medulloblastomas, oligodendrogliomas, meningiomas, and schwannomas. The presence of the ALT phenotype in GBM has been described previously,[15,25–27] but all cases assessed were in adults. The prevalence of the ALT phenotype in adult GBM cases in the present study (11%) is similar to that in a recently published large retrospective series using the same assay (15%).[27] Previous studies on smaller sets of adult GBM reported prevalence at 22% (7/32)[15] and 25% (19/77).[26] Notably, Henson et al[15] observed an inverse relationship between ALT-positivity and patient age, and this observation was confirmed by McDonald et al[27] in a retrospective cohort. Here, we report a significantly increased prevalence in pediatric GBM (44%), compared with adult GBM (11%). In adult GBM, recent results have shown a significantly longer overall survival in patients with mutations of the isocitrate dehydrogenase 1 gene (*IDH1*) in the presence of ALT.[27] It would be worthwhile to examine the prognostic associations of ALT in pediatric GBM, although these tumors almost never show mutations in *IDH1*.

We identified many tumor types that apparently may not use the ALT pathway for telomere maintenance. In particular, we assessed hundreds of cases of adenocarcinomas arising from the prostate, colon, pancreas, and small intestine and did not observe a single ALT-positive tumor. These results suggest that particular tumor types preferentially use telomerase activation for stabilization of telomeres and emphasize the previous findings that the ALT phenotype is more common in tumors with mesenchymal and neuroepithelial cell origins.[12,16] The ability of epithelial cells to up-regulate telomerase more easily than mesenchymal cells may account for these differences.

In sarcomas, the ALT phenotype has been described previously in specific subtypes.[16] In agreement with these reports,[16,28] we found the ALT phenotype in 24% of liposarcomas, 53% of leiomyosarcomas, and 63% of undifferentiated pleomorphic sarcomas (which includes malignant fibrous histiocytomas). In contrast to GBM, in liposarcomas the presence of the ALT phenotype confers a poor prognosis.[28,29] Other genetic changes associated with the ALT phenotype presumably play important roles in determining the prognostic significance within a given tumor type. Although the ALT phenotype is extremely prevalent in certain sarcomas, we did not observe ALT in Ewing's sarcomas/primitive neuroectodermal tumors, gastrointestinal stromal tumors, or Kaposi's sarcomas. These findings are consistent with previous investigations showing that sarcomas characterized by specific chromosomal translocations tend to maintain telomeres via telomerase activation, whereas sarcomas with complex karyotypes are capable of using the ALT pathway.[14,30]

As outlined above, the results presented in the current study are in broad agreement with previous reports on ALT in human cancers.[16] However, there is a major difference in one previously assessed cancer subtype: we did not observe the ALT phenotype in gastric carcinomas ($n = 155$). This is in contrast to findings of Omori et al,[31] who recently reported a 38% overall ALT prevalence in gastric carcinomas, with an even higher prevalence reported in gastric carcinomas with microsatellite instability. One potential explanation for these discrepant results is that the previous study included an amplification protocol to intensify the telomere FISH signals, whereas in the current study we assessed telomere signals directly, without the use of amplification. Thus, it is possible that signal amplification in the prior study may have resulted in overly bright telomere FISH intensities, causing them to be mistaken for ALT-associated telomeric foci.

Despite the large number of cases and cancer subtypes assessed, the present study has limitations. Because of the TMA sampling methodology, prevalence estimates may underestimate the true prevalence of the ALT phenotype in some cancer subtypes. In addition, estimates of the prevalence of the ALT phenotype in certain subtypes may be too high or too low, particularly if the absolute numbers of cases examined are small. However, the present findings may help guide future studies to determine the actual prevalence of ALT within a given cancer subtype. All of the cases assessed in this survey were primary tumors; therefore, future studies are needed to assess the prevalence of ALT in metastatic lesions. Similarly, we assessed only a small number of premalignant lesions, and the role of the ALT phenotype in cancer progression is not yet elucidated. Although the present study comprehensively covered many cancer subtypes, some subtypes were not assessed; in particular, no leukemia cases were assessed. Finally, although several telomerase antibodies are available, none have been adequately validated for use in formalin-fixed, paraffin-embedded tissues. Thus, the use of such fixed specimens in the present study precluded additional analyses that would have required either fresh or frozen tissue samples, such as assessment of telomerase enzymatic activity.

The ALT telomere maintenance mechanism provides prognostic information in some cancers.[16] Further studies are needed to assess the prognostic significance and unique biology of tumors that express ALT. The present study offers a springboard to guide future investigations in large cohorts that specifically focus on the tumor types exhibiting ALT to determine the true prevalence and potential prognostic value of this phenotype. Finally, these results may have therapeutic consequences, given that cancers using the ALT pathway are predicted to be resistant to anti-telomerase therapies, some of which have entered phase I/II clinical trials. Further understanding of the molecular mechanisms of ALT will be paramount in designing novel anti-cancer therapeutics targeting the ALT pathway.

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Title: ALTERNATIVE LENGTHENING OF TELOMERES IN HUMAN CARCINOMA SUBTYPES

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Background: Approximately 10-15% of human cancers do not show evidence of telomerase activity, and a subset of these maintain telomere lengths by a genetic recombination-based mechanism termed alternative lengthening of telomeres (ALT). The ALT phenotype, relatively common in certain sarcomas and germ cell tumors, has only rarely been reported in carcinomas. It has been suggested that telomerase expression is more stringently suppressed in mesenchymal tissues, potentially explaining the higher frequency of ALT in sarcomas; however, the prevalence of ALT has not been thoroughly assessed in carcinomas. Our lab recently reported ALT in a small subset of breast carcinomas; it has also been detected in some adrenal cortical carcinomas. The purpose of this study was to systematically investigate the frequency of ALT in epithelial tumors.

Design: We analyzed tissue microarrays (TMA) of carcinomas of breast (n= 116), salivary gland (n=31), lung (n=197), liver (n=91), kidney (n=114), ovarian serous (n=46), stomach (n=89), colon (n=104), small intestine (n=195), pancreas (n=432), and esophagus (n=88). The arrays included an assortment of primary and metastatic tumors. A sarcoma TMA (n=36) was used as a positive control. Telomere lengths were directly assessed using fluorescence in situ hybridization (FISH) with combined promyelocytic leukemia (PML) protein immunofluorescence.

Result: The ALT phenotype was identified in 7 of 91 primary liver carcinomas and 2 of 114 conventional renal cell carcinomas (1 primary, 1 metastatic). A fourth ALT-positive case was identified in a primary breast carcinoma, in addition to the 3 previously reported from our lab. In summary, 1,503 total carcinomas were analyzed yielding 13 cases of ALT (overall frequency = 0.86%).

Conclusion: This is the first observation of the ALT phenotype in liver and kidney tumors. ALT is very rare in carcinomas overall; observation in our study was restricted to 3 tumor types (breast, liver, and kidney). The telomere maintenance mechanism confers a poor prognosis in some cancers; further studies are needed to assess the prognostic significance and unique biology of carcinomas that express ALT. As cancers using the ALT pathway are predicted to be resistant to therapies based on telomerase inhibition, these results may have therapeutic consequences.

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Title: **PREVALENCE OF THE ALTERNATIVE LENGTHENING OF TELOMERES (ALT) TELOMERE MAINTAINENCE MECHANISM IN HUMAN CANCER SUBTYPES**

Abstract: Approximately 10-15% of human cancers lack detectable telomerase activity and a subset of these maintain telomere lengths by the telomerase-independent telomere maintenance mechanism termed alternative lengthening of telomeres (ALT). The ALT phenotype, relatively common in subtypes of sarcomas and astrocytomas, has rarely been reported in epithelial malignancies. However, the prevalence of ALT has not been thoroughly assessed across all cancer types. Thus, we comprehensively surveyed the ALT phenotype in a broad range of human cancers. In total, two independent sets comprising 6,110 primary tumors from 94 different cancer subtypes, 541 benign neoplasms and 264 normal tissue samples were assessed by combined telomere-specific fluorescence *in situ* hybridization and immunofluorescence labeling for PML protein. Overall, ALT was observed in 3.73% (228/6,110) of all tumor specimens and was not observed in benign neoplasms or normal tissues. This is the first description of ALT in carcinomas arising from the bladder, cervix, endometrium, esophagus, gallbladder, kidney, liver and lung. Additionally, this is the first report of ALT in medulloblastomas, oligodendrogliomas, meningiomas, schwannomas and pediatric glioblastoma multiformae. Previous studies have shown associations between ALT status and prognosis in some tumor types; thus, further studies are warranted to assess the potential prognostic significance and unique biology of ALT-positive tumors. These findings may have therapeutic consequences, since ALT-positive cancers are predicted to be resistant to anti-telomerase therapies.

Altered Telomeres in Tumors with *ATRX* and *DAXX* Mutations

Christopher M. Heaphy,[1*†] Roeland F. de Wilde,[1*] Yuchen Jiao,[2*] Alison P. Klein,[1,3,4] Barish H. Edil,[3] Chanjuan Shi,[5] Chetan Bettegowda,[2,6] Fausto J. Rodriguez,[1] Charles G. Eberhart,[1] Sachidanand Hebbar,[1] G. Johan Offerhaus,[7] Roger McLendon,[8] B. Ahmed Rasheed,[8] Yiping He,[8] Hai Yan,[8] Darell D. Bigner,[8] Sueli Mieko Oba-Shinjo,[9] Suely Kazue Nagahashi Marie,[9] Gregory J. Riggins,[6] Kenneth W. Kinzler,[2] Bert Vogelstein,[2] Ralph H. Hruban,[1,3] Anirban Maitra,[1,3] Nickolas Papadopoulos,[2‡] Alan K. Meeker[1,3‡]

A recent study of pancreatic neuroendocrine tumors (PanNETs) revealed that 43% harbored inactivating mutations in the *ATRX* or *DAXX* genes (*1*). The proteins encoded by *ATRX* and *DAXX* interact with one another and play multiple cellular roles, including chromatin remodeling at telomeres, where they are required for the incorporation of the histone variant H3.3 (*2*–*6*). Given the potential role of ATRX and DAXX in modulating telomeric chromatin, we evaluated telomere status in PanNETs in which *ATRX* and *DAXX* mutational status had been determined through Sanger sequencing. Telomere-specific fluorescence in situ hybridization (FISH) revealed that 25 of 41 (61%) PanNETs displayed large, ultrabright telomere FISH signals, a nearly universal feature of the telomerase-independent telomere maintenance mechanism termed alternative lengthening of telomeres (ALT) (Fig. 1) (*7*). *ATRX* and *DAXX* gene mutations both were significantly correlated with ALT positivity ($P < 0.008$ for each gene). All 19 (100%) PanNETs with *ATRX* or *DAXX* gene mutations were ALT-positive (table S1), whereas 6 of 20 cases without detectable mutations were ALT-positive. Subsequent immunolabeling revealed that each of the six ALT tumors lacking point mutations or insertions or deletions had lost nuclear expression of either ATRX or DAXX (Fig. 1, fig. S1, and table S1). In contrast, the 16 tumors without ALT showed robust nuclear labeling for both proteins (table S1), and this relationship was statistically significant ($P = 0.012$ and $P = 0.003$, respectively). Thus, there was a perfect correlation between inactivation of ATRX or DAXX and the ALT phenotype in PanNETs.

To ascertain whether *ATRX* and *DAXX* gene mutations might be more generally associated with the ALT phenotype, we examined 439 tumors of other types. We did not identify any *DAXX* mutations but did identify *ATRX* mutations in cancers of the central nervous system (CNS): pediatric glioblastoma multiforme (GBM) (14.3%), adult GBM (7.1%), oligodendrogliomas (7.7%), and medulloblastomas (1.5%) (Fig. 1 and table S2). To determine whether the ALT status of the CNS tumors was correlated with the presence of *ATRX* mutations, we performed telomere FISH on eight *ATRX* mutant cases in which tumor material was available. In each of these eight cases, extremely bright telomeric foci were identified in the neoplastic cells, and immunolabeling showed loss of nuclear expression of ATRX (Fig. 1 and table S3). We concurrently performed telomere FISH on 16 cases of the same histologic subtypes without detectable mutations of *ATRX* or *DAXX* and found that none had evidence of abnormal telomere foci.

We also studied the human osteosarcoma cell line U-2 OS because this line was a prototype for delineating the ALT phenotype (*8*). We found that exons 2 to 19 of *ATRX* were homozygously deleted in these cells, inactivating the gene product and causing a lack of ATRX immunolabeling (fig. S2).

There is thus a strong correlation between inactivation of *ATRX* or *DAXX* and the ALT phenotype in unrelated tumor types. Previous evidence suggests that the ATRX-DAXX complex functions in heterochromatin assembly at repetitive G-rich regions, such as telomeres (*3*, *5*, *6*). Furthermore, decreasing ATRX or H3.3 in mouse embryonic stem cells results in telomere destabilization and up-regulation of telomere repeat-containing RNA (*6*, *9*–*11*).

Our results are consistent with a model in which loss of ATRX-DAXX function impairs the heterochromatic state of the telomeres, perhaps because of reduced levels of H3.3 incorporation, leading to telomere destabilization and increased HR at the telomeres and thereby facilitating the development of ALT.

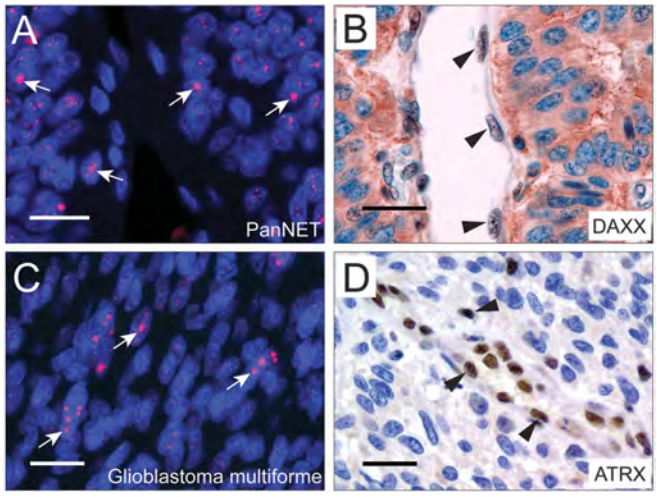

Fig. 1. Representative images of ALT-positive tumors with ATRX or DAXX mutations. (**A**) Example of ALT-positive PanNET. Large, ultrabright telomere FISH signals (red) indicative of ALT are marked (arrows). (**B**) Immunolabeling of the same PanNET shows loss of nuclear DAXX protein in tumor cells. (**C**) Example of ALT-positive GBM. Large, ultrabright telomere FISH signals (red) indicative of ALT are marked (arrows). (**D**) Immunolabeling of the same GBM shows loss of nuclear ATRX protein in tumor cells. In (B) and (D), benign endothelial cells (arrowheads) served as positive immunostaining controls. Scale bars, 30 μm.

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Supporting Online Material

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Altered Telomeres in Tumors with *ATRX* and *DAXX* Mutations

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This PDF includes:

Supplementary materials and methods

Tissue Samples and Mutational Analysis

In order to assess the potential consequences of *ATRX* and *DAXX* gene mutations on telomeres, 41 sporadic, nonfunctional PanNETs were chosen from a series of PanNETs whose *ATRX* and *DAXX* gene mutational status was previously known (1). Of the 41 PanNETs examined, 9 had *ATRX* gene mutations, 10 had *DAXX* gene mutations, and the remaining 22 did not have any point mutations or indels in the *ATRX* and *DAXX* genes. Clinical information on the patients evaluated in this study was obtained from the Johns Hopkins Hospital in the context of approved IRB protocols. The *ATRX* and *DAXX* genes were also sequenced in 65 cases of medulloblastoma, 13 cases of oligodendroglioma, 133 cases of glioblastoma multiformae (21 pediatric GBM and 112 adult GBM), 11 neuroblastomas, 25 ovarian carcinomas, 96 breast carcinomas and 96 pancreatic ductal adenocarcinomas. The mutation frequencies, as well as details on the specific mutations in these tumors are presented in tables S2 and S3, respectively.

Immunohistochemistry

Immunolabeling for the ATRX and DAXX proteins was performed on formalin-fixed, paraffin embedded sections as previously described (1). Briefly, heat-induced antigen retrieval was performed in a steamer using citrate buffer (catalog# H-3300, Vector Laboratories) for 30 minutes. Endogenous peroxidase was blocked (catalog# S2003, Dako) and serial sections were then incubated with primary antibody; anti-ATRX (1:400 dilution; catalog# HPA001906, Sigma-Aldrich, lot R00473) or anti-DAXX (1:150 dilution;

catalog# HPA008736, Sigma-Aldrich, lot A39105) for 1 hour at room temperature. The primary antibodies were detected by 30 minute incubation with HRP-labeled secondary antibody (catalog# PV6119, Leica Microsystems) followed by detection with 3,3′-Diaminobenzidine (Sigma-Aldrich), counterstaining with Harris hematoxylin, rehydration and mounting. Only nuclear labeling of either protein was evaluated. The immunolabeled PanNET slides were assessed and scored by 2 authors (R.H. and A.M.); the immunolabeled CNS tumor slides were assessed by F.R. Internal controls included endothelial cells (including within intra-tumoral vessels), islets of Langerhans in PanNETs and neurons in CNS tumors, which demonstrated strong nuclear immunolabeling for both ATRX and DAXX.

Telomere-specific FISH and microscopy

Combined telomere-specific FISH and immunofluorescence labeling for ATRX, DAXX and PML was conducted as previously described (12, 13). Briefly, deparaffinized slides were hydrated, steamed for 20 minutes in citrate buffer (catalog# H-3300; Vector Laboratories), dehydrated and hybridized with a Cy3-labeled peptide nucleic acid (PNA) probe complementary to the mammalian telomere repeat sequence ([N-terminus to C-terminus] CCCTAACCCTAACCCTAA). As a positive control for hybridization efficiency, a FITC-labeled PNA probe having specificity for human centromeric DNA repeats (ATTCGTTGGAAACGGGA; CENP-B binding sequence) was also included in the hybridization solution (14). Following post-hybridization washes, the desired primary antibody was applied (anti-ATRX, as described above; anti-DAXX, as described above; anti-PML antibody, 45 minute incubation at 1:100 dilution; catalog# PG-M3, DAKO),

followed by application of species-appropriate Alexa 488 fluorescent secondary antibody (Molecular Probes Cat.# A-11034 and A-11001) and nuclear counterstaining with DAPI . Slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) and appropriate fluorescence excitation/emission filters. Grayscale images were captured for using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged. Quantification from the digital images was conducted using Telometer, a custom software plugin created for the open source image analysis program ImageJ, freely available for download (http://bui2.win.ad.jhu.edu/telometer/).

The FISH and immunolabeled slides were assessed and scored independently by 2 authors (C.H. and A.K.M.). Large, ultra-bright telomere repeat DNA aggregates are unique to ALT-positive cell populations and are significantly larger and brighter than the FISH signals emanating from individual telomeres in the same cell population (for further details, see detailed discussion of these foci as surrogate markers for the ALT phenotype below).

In our study, PanNETs were classified as ALT-positive if they met the following criteria: (i) the presence of ultra-bright, intra-nuclear foci of telomere FISH signals, with integrated total signal intensities for individual foci being > 10 fold that of the per cell mean integrated signal intensities for all telomeric signals in individual benign stromal cells within the same case (Quantitative data on representative individual telomere aggregates in 10 randomly chosen PanNET cases is presented in table S4); (ii) ≥1% of neoplastic cells displaying ALT-associated telomeric DNA foci. Tumor samples lacking

ALT-associated telomeric foci in which at least 5000 cells were assessed were considered ALT-negative. In all cases, areas exhibiting necrosis were excluded from consideration.

Statistical Analyses

Fisher's Exact Test was used to assess the relationship between ALT phenotype and mutational status and immunolabeling. STATA v11 was used for all statistical analysis (reference: StataCorp. 2009. *Stata Statistical Software: Release 11*. College Station, TX: StataCorp LP).

Supplemental Text

Telomere-specific FISH as a surrogate marker of the ALT phenotype

In considering the various assays available for identifying ALT, we chose to use the presence of large, abnormally intense, intra-nuclear telomere DNA FISH signals. Our rationale for this choice is outlined below.

ALT was originally discovered in immortalized human cell lines *in vitro*, with the defining characteristics of long-term telomere maintenance in the absence of telomerase enzymatic activity. However, in subsequent studies, it has been shown that the measurement or either telomerase expression or telomerase activity in un-fixed human tumor specimens is not a reliable indicator of ALT status, as several instances have been reported of tumors in which both telomere maintenance mechanisms (TMMs) are present (8, 15-17). Furthermore, significant intra-tumoral heterogeneity in telomerase levels has been observed and telomerase activity assays are susceptible to

the confounding effects of contaminating telomerase-positive normal cells (e.g. activated lymphocytes) capable of producing false positives. Conversely, a lack of telomerase does not necessarily mean a tumor is ALT-positive as many cases show no apparent TMM at all (17, 18). Thus, in addition to the requirement for fresh or frozen tissue samples, telomerase assays are not sufficiently specific to allow for reliable assessment of ALT status in a given tumor.

As pointed out in a recent comprehensive review on ALT assays by Henson and Reddel, several other surrogate markers for the ALT phenotype, including telomeric sister chromatin exchange rates, instability of specific minisatellites, telomere-specific Southern blotting of high molecular weight genomic DNA, and detection of telomeric circular DNA species, are also lacking in specificity and/or require actively growing cells or un-fixed tissue samples (19).

Telomere-specific FISH is readily applicable to FFPE tissue specimens. When performed on cells and tissues defined as being ALT-positive by other independent techniques, telomere FISH invariably reveals the presence of large, abnormally bright, aggregates of telomere DNA repeats within interphase nuclei of ALT-positive cells. In the original report from Roger Reddel's group, these aggregates were found to be perfectly specific for ALT, being present in all ALT-positive immortalized cell lines and ALT-positive tumor-derived cell lines examined, as well as all ALT-positive human tumor tissue samples tested (7). Conversely, such aggregates were not observed in any ALT-negative cell line or tumor sample. Thus, these abnormal aggregates, which are highly distinctive, were shown to be completely specific for the ALT phenotype. The Reddel group later went on to show that these aggregates appeared to be contained in

a subset of PML nuclear bodies, dubbed ALT-associated PML bodies (APBs), and the identification of APBs through combined telomere FISH and immunofluorescence (IF) for PML protein was proposed as an assay for the ALT phenotype in fixed tissue specimens (20). However, since the publication of this report, cells exhibiting phenotypic hallmarks of ALT, including telomere DNA aggregates, but lacking the characteristic PML co-staining that marks APBs have been reported (21-23). These observations clearly indicate that APBs are not universal markers of the ALT phenotype. Furthermore, in human tumors, the APB assay has only been validated in relatively small subsets of soft tissue sarcomas and astrocytomas.

We carefully assessed co-staining for PML protein by combined PML immunofluorescence (IF) and telomere-specific FISH on all of the fixed tissue specimens used in our study. PML nuclear bodies were readily visualized by PML IF in the stromal cells within the tumor sections, thus providing internal positive controls for the PML immunostaining. Although we found that PML protein frequently co-localized with large, ultra-bright, telomere FISH signals, (see representative APB images in Fig. S3), there were also examples of ultra-bright FISH signals that did not co-stain with PML protein. The details are as follows: in 39% of ALT-positive cases, ultra-bright FISH signals co-stained with PML protein, in 36% of cases, ultra-bright FISH signals did not co-stain with PML protein, and in the remaining 25% of cases, we observed intra-tumoral heterogeneity of co-staining, with some ultra-bright FISH signals co-staining with PML protein but others lacking such co-stain.

Considering the published examples of ALT in the absence of APBs, the somewhat limited validation of the APB assay, and the fact that telomere aggregates by

themselves appear to be nearly universal surrogate markers of ALT, we diagnosed ALT based on the observation of large, ultra-bright, intra-nuclear foci of telomeric DNA, regardless of the presence or absence of co-localized PML protein. The following additional points support our reliance on this marker for diagnosing ALT: (i) The abnormally bright FISH signals lacking PML were otherwise indistinguishable from those that were PML-associated; (ii) The abnormal FISH signals were only observed in cancer cell nuclei, never in the surrounding normal cells; (iii) With the exception of PanNETs, in which telomeres had never before been evaluated, abnormal FISH signals were only found in tumor types (e.g. GBM, MB) previously shown to utilize the ALT; (iv) The quantitative FISH data obtained using image analysis on representative cases (supplementary table S4) indicate that each one of the large, abnormal FISH signals represent several-fold more telomeric DNA than is contained within an entire normal cell's nucleus; and thus cannot be due merely to spatial proximity of several telomeres, nor to a single long telomere; however, they are fully compatible with the type of telomere DNA aggregates previously described to be associated with ALT-positive cells; and (v) To our knowledge, there are no known counter-examples in the literature of cells displaying large, ultra-bright, abnormal intra-nuclear telomere FISH foci that are not ALT-positive.

In summary, we consider the detection of abnormal telomere DNA aggregates via telomere-specific FISH to be the most sensitive and specific single surrogate marker available for identifying the ALT phenotype in fixed tissue specimens.

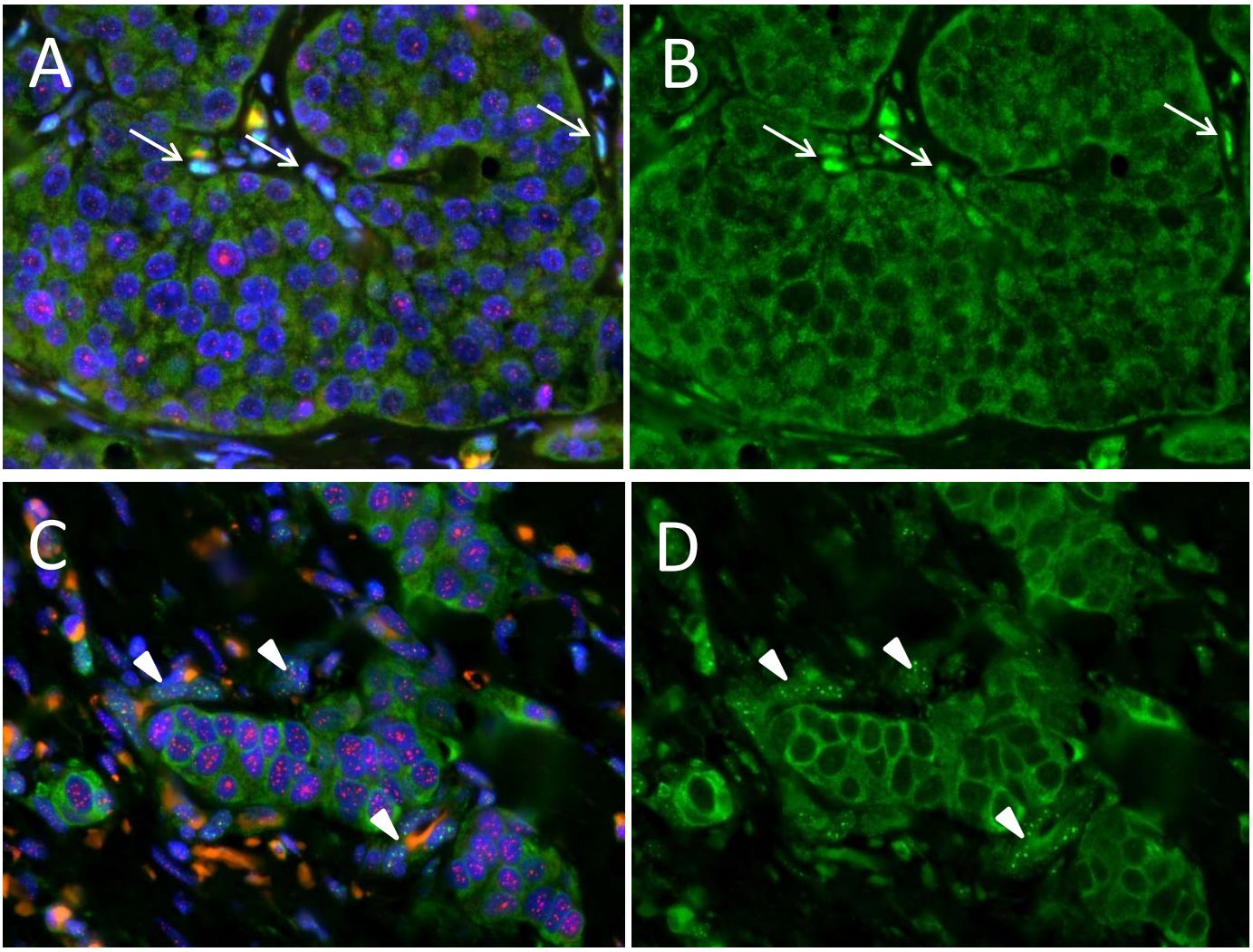

Figure S1 Telomere-FISH and immunofluorescence co-staining in ALT-positive tumors. (**A**) ALT-positive PanNET co-stained with telomere FISH (red) and ATRX protein (green). (**B**) same image as in A, omitting telomere and DAPI channels, highlighting the loss of nuclear ATRX. Benign stromal cells positive for nuclear ATRX protein (internal positive staining controls) are indicated by arrows. (**C**) ALT-positive PanNET co-stained with telomere FISH (red) and DAXX protein (green). (**D**) same image as in C, omitting telomere and DAPI channels. Punctate nuclear DAXX staining in benign stromal cells is indicated by arrow heads. Original magnification=400X

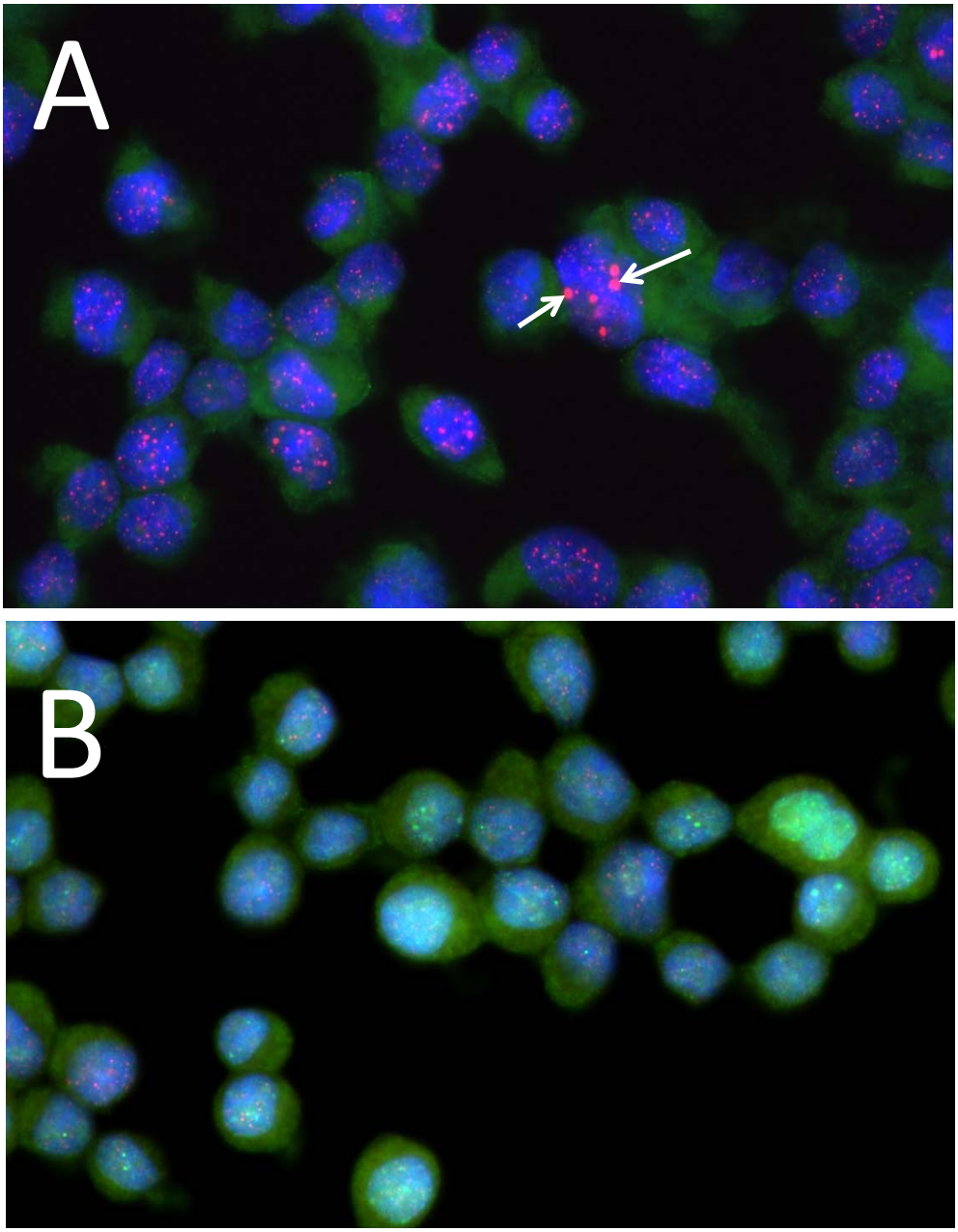

Figure S2 Combined telomere-FISH and co-staining for ATRX protein by immunofluorescence. (**A**) ALT-positive osteosarcoma-derived cell line U2-OS showing lack of nuclear ATRX protein (green) and ALT-associated bright telomere DNA foci (arrows). The green cytoplasmic signal in U2-OS seen here was also present in control samples in which the primary anti-ATRX antibody was omitted; thus it is due to autofluorescence (**B**) ALT-negative PanNET cell line BON-1, showing ATRX nuclear-positivity (green). In both cases telomere FISH signals (red) and nuclear DNA counterstained with DAPI (blue) are also shown. Original magnification= 400X for both images.

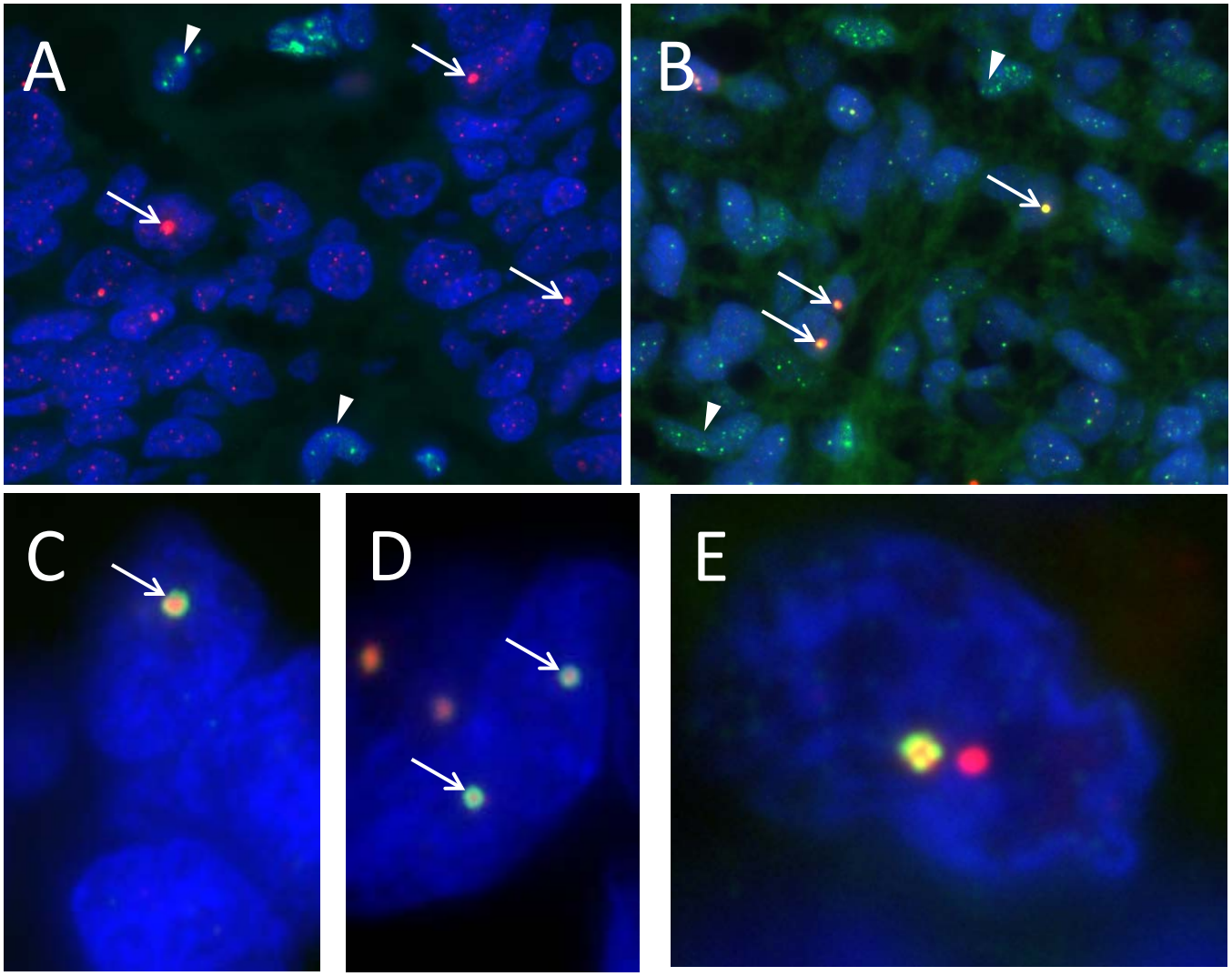

Figure S3 Telomere-FISH and immunofluorescence co-staining of PML protein in ALT-positive tumors.

(**A**) ALT-positive PanNET co-stained with telomere FISH (red) and PML protein (green): co-localization of PML protein and large telomere DNA aggregates (arrows) is lacking in this case. PML nuclear bodies in benign stromal cells (arrowheads) act as internal staining controls for PML protein (**B**) ALT-positive PanNET exhibiting co-staining of PML protein (green) and telomere DNA foci (red), arrowheads: PML nuclear bodies in benign stromal cells. (**C, D**) Higher magnification of co-localized PML protein (green) and large telomere DNA foci (red). (**E**) Single ALT-positive PanNET tumor cell with two ALT-associated telomere foci (red); one with and one without co-localizing PML protein (green). In all cases, nuclear DNA has been counter stained with DAPI (blue). A-D, original magnification=400X. E, original magnification=1000X.

Table S1. PanNET cases grouped according to mutation status for ATRX and DAXX and displaying ALT-status and immunohistochemistry of ATRX and DAXX

Gene Status	Case	ALT-status*	ATRX IHC†	DAXX IHC†
ATRX Mutation	PanNET5	***Pos***	***Neg***	Pos
	PanNET13	***Pos***	***Neg***	Pos
	PanNET27	***Pos***	***Neg***	Pos
	PanNET35	***Pos***	***Neg***	Pos
	PanNET52	***Pos***	***Neg***	Pos
	PanNET59	***Pos***	Pos	Pos
	PanNET78	***Pos***	Pos	Pos
	PanNET85	***Pos***	***Neg***	Pos
	PanNET112‡	***Pos***	***Neg***	Pos
DAXX Mutation	PanNET25	***Pos***	Pos	***Het***
	PanNET31	***Pos***	Pos	***Het***
	PanNET44	***Pos***	Pos	***Neg***
	PanNET56	***Pos***	Pos	***Neg***
	PanNET77	***Pos***	Pos	***Neg***
	PanNET84	***Pos***	Pos	***Het***
	PanNET87	***Pos***	Pos	***Het***
	PanNET93	***Pos***	Pos	***Neg***
	PanNET104	***Pos***	Pos	***Neg***
	PanNET133	***Pos***	Pos	***Neg***
ATRX/DAXX WT	PanNET6	Neg	Pos	Pos
	PanNET10	Neg	Pos	Pos
	PanNET21	Neg	Pos	Pos
	PanNET24	Neg	Pos	Pos
	PanNET29	***Pos***	Pos	***Het***
	PanNET36	Neg	Pos	Pos
	PanNET39	***Pos***	***Neg***	Pos
	PanNET45	***Pos***	Pos	***Neg***
	PanNET57	Neg	Pos	Pos
	PanNET61	***Pos***	***Neg***	Pos
	PanNET63	Neg	Pos	Pos
	PanNET64	***Pos***	***Het***	Pos
	PanNET66	Neg	Pos	Pos
	PanNET69	Neg	Pos	Pos
	PanNET79	Neg	Pos	Pos
	PanNET80	***Pos***	Pos	***Neg***
	PanNET83	Neg	Pos	Pos
	PanNET91	Neg	Pos	Pos
	PanNET121	Neg	Pos	Pos
	PanNET126	Neg	Pos	Pos
	PanNET128	Neg	Pos	Pos
	PanNET129	Neg	Pos	Pos

* The intensity of telomere FISH signals was assessed to be either negative or positive for the ALT phenotype.

† Immunohistochemistry was scored as uniformly positive, negative or heterogeneous for nuclear labeling.

‡ Multifocal tumor, featuring ALT and negative nuclear immunolabeling in the majority of the tumor.

Table S2. Human Cancers Sequenced for *ATRX* and *DAXX*

Cancer Type	Number of samples analyzed	Number of samples with mutations*	Prevelance
Pediatric Glioblastoma	21	3	14.3%
Adult Glioblastoma	112	8	7.1%
Oligodendroglioma	13	1	7.7%
Medulloblastoma	65	1	1.5%
Neuroblastoma	11	0	0.0%
Ovarian	25	0	0.0%
Breast	96	0	0.0%
Pancreas	96	0	0.0%

*Mutations were identified only in *ATRX*

Table S3. Mutations in *ATRX* in Human CNS Tumors.

Sample	Cancer type	Nucleotide (genomic)*	Amino acid[$] (protein)	Mutation type	ALT
MB116PT	Medulloblastoma	g.chrX:76778161_76778162insA	frameshift	indel	yes
OLID 04 PT	Oligodendroglioma	g.chrX:76824745_76824748delTCTC	frameshift	indel	yes
PGBM 09 PT	Pediatric Glioblastoma	g.chrX:76741670_76741673delCTAT	frameshift	indel	yes
PGBM 12 PT	Pediatric Glioblastoma	g.chrX:76798738_76798741delACTA	frameshift	indel	yes
GLI-02X	Pediatric Glioblastoma	g.chrX:76798774_76798775delAG(hom)	frameshift	indel	ND
NT190	Adult Glioblastoma	g.chrX:76665385C>A	frameshift	splice-site	ND
Yan104X	Adult Glioblastoma	g.chrX:76825188_76825194delTTGAGGA	frameshift	indel	ND
Yan106X	Adult Glioblastoma	g.chrX:76831065delG(hom)	frameshift	indel	yes
Yan123X	Adult Glioblastoma	g.chrX:76806828_76806829insT	frameshift	indel	ND
Yan211T	Adult Glioblastoma	g.chrX:76825743_76825744delTG	frameshift	indel	yes
Yan129X	Adult Glioblastoma	g.chrX:76826615C>T(hom)	p.W263X	nonsense	ND
Yan237T	Adult Glioblastoma	g.chrX:76700843G>A(hom)	p.R2153C	missense	yes
Yan238T	Adult Glioblastoma	g.chrX:76760970C>T	p.R1803H	missense	yes

*Coordinates refer to human reference genome hg18 release (NCBI 36.1, March 2006).

$ Single-letter abbreviations for the amino acid residues are as follows: C, Cys; H, His; R, Arg; W, Trp; and X, STOP.

(hom): these mutations appear homozygous

ND: not determined because tumor tissue unavailable.

Table S4. Quantification of ALT-associated telomere DNA-specific FISH signals.

Case	Normal cell mean total telomere FISH intensity	PanNET ETBF FISH intensity	Ratio of PanNET EBTF to normal cell telomere content
PanNET5	40319	1090480	27
PanNET13	14566	1670256	115
PanNET25	25925	527400	20
PanNET27	39959	940662	24
PanNET29	16534	900828	54
PanNET31	32726	2185140	67
PanNET35	13292	3418500	257
PanNET52	23902	735480	31
PanNET59	18318	1715957	94
PanNET93	170013	10510700	62

Telomere FISH signals were quantified from individual extremely bright telomere foci (EBTF) in a representative subset (n=10) of ALT-positive PanNET tissue samples. Quantitative data was obtained using grey scale monochrome images and the custom image analysis program Telometer (http://bui2.win.ad.jhu.edu/telometer/). Short exposure times were used for image collection of EBTF to ensure that these FISH signals fell within the linear range of the camera. Telomere DNA-specific FISH signals were likewise quantified for all telomeres within individual normal stromal cells within the same tissue samples (n=10 cells per specimen). These stromal cell telomere FISH signals were then summed, giving an intensity value representing the total telomere intensity per cell. These per cell total telomere intensity values were then averaged within a given PanNET tissue sample. Intensity ratios were calculated by dividing the normalized telomere FISH signals for individual EBTF by the average per cell sum of individual telomere FISH signals for the benign stromal cells within the same PanNET case, with appropriate linear adjustment for differences in exposure times during image collection.

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The potential utility of telomere-related markers for cancer diagnosis

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Abstract

The role telomeres and telomerase play in the initiation and progression of human cancers has been extensively evaluated. Telomeres are nucleoprotein complexes comprising the hexanucleotide DNA repeat sequence, TTAGGG and numerous telomere-associated proteins, including the six member Shelterin complex. The main function of the telomere is to stabilize the ends of the chromosomes. However, through multiple mechanisms, telomeres can become dysfunctional, which may drive genomic instability leading to the development of cancer. The majority of human cancers maintain, or actively lengthen, telomeres through up-regulation of the reverse transcriptase telomerase. Because there are significant differences in telomere length and telomerase activity between malignant and non-malignant tissues, many investigations have assessed the potential to utilize these molecular markers for cancer diagnosis. Here, we critically evaluate whether measurements of telomere lengths and telomerase levels may be clinically utilized as diagnostic markers in solid tumours, with emphasis on breast and prostate cancer as representative examples. Future directions focusing on the direct detection of dysfunctional telomeres are explored. New markers for telomere dysfunction may eventually prove clinically useful.

Keywords: breast cancer • cancer • detection • diagnosis • prostate cancer • telomerase • telomere • telomere dysfunction

Introduction

Telomeres are nucleoprotein complexes located at the extreme ends of eukaryotic chromosomes [1]. In normal human somatic cells, telomeres comprise 5–12 kb of the repeating hexanucleotide DNA sequence, TTAGGG [2, 3]. Numerous proteins are associated with these repetitive regions. The Shelterin complex, a core set of six proteins integral for telomere function, is composed of telomeric repeat binding factor (TRF)1, TRF2, protection of telomeres 1 (POT1), TRF interacting protein 1 (TIN2), repressor/activator protein 1 (RAP1) and TPP1 [4, 5]. The telomere complex primarily functions to mask double strand break DNA damage signals at telomeres, inhibit exonucleolytic degradation and prevent chromosomal fusions [6, 7].

Telomeres can be critically shortened by incomplete replication of the lagging strand during DNA synthesis, known as the 'end-replication problem' [8, 9]. Through this process, each telomeric end shortens by approximately 50–100 base pairs during each successive cell division. Other known mechanisms leading to telomere loss include oxidative DNA damage [10, 11] and alterations of Shelterin proteins [12]. In normal somatic cells, significant telomere shortening leads to p53-dependent senescence

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or apoptosis [13, 14]. As a result, there is a limited number of population doublings a somatic cell may undergo before entering a senescent state. In cancer cells, these cell cycle checkpoints are abrogated, for example through mutations in tumour suppressor proteins. Consequently, unchecked cellular proliferation continues and genomic instability may ensue *via* chromosomal breakage–fusion–bridge cycles [15].

In the vast majority (85–90%) of human cancers, telomere length appears to be maintained, or actively lengthened, through up-regulation of the enzyme telomerase. Telomerase is a reverse transcriptase that has the ability to synthesize new telomere DNA using an internal RNA template [1, 16, 17]. Telomerase is minimally composed of two components, the telomerase reverse transcriptase (TERT) protein [human telomerase reverse transcriptase (hTERT)] and the telomerase RNA template component [human telomerase RNA (hTR)] [18–22]. Because hTR is ubiquitously expressed, hTERT is considered the rate-limiting component that determines telomerase activity. Telomere loss may also be compensated in some cancers, by the telomerase-independent alternative lengthening of telomeres (ALT) pathway [23].

The basic biology of telomeres and telomerase has been a focus of research for decades and mounting evidence demonstrates the crucial role telomere biology plays in the initiation and progression of carcinogenesis. Previous reviews have discussed the potential prognostic significance of telomere and telomerase measurements in solid tumours [24, 25] and haematological malignancies [26, 27]. Here, we critically assess whether measurements of telomere lengths and/or telomerase levels will be useful as diagnostic markers for solid tumours. Due to space limitations, we focus predominantly on two common malignancies, breast and prostate cancer, and provide specific examples for other cancer types.

Methods for telomere length and telomerase detection

Numerous methods have been developed to measure either actual telomere length or total relative telomere content, a proxy for mean length. These methods include terminal restriction fragment (TRF) Southern blot analysis [28, 29], quantitative fluorescence *in situ* hybridization (Q-FISH) [30–32], Flow-FISH [33], slot blot assay [34, 35], quantitative telomere-specific PCR (Q-PCR) [36, 37] and single telomere length analysis (STELA) [38]. Likewise, measurement of telomerase enzymatic activity or telomerase gene expression in human biological samples, either in tissue or other bodily fluids, can be performed by different methods. These methods include telomere repeat amplification protocol (TRAP) [39] or detection of transcript levels of hTERT or hTR, either by RT-PCR or *in situ* hybridization. The potential strengths and limitations of each assay are summarized in Table 1.

Telomere length as a potential diagnostic marker in cancer

Breast cancer

Mirroring similar observations in most other cancers, initial studies measuring bulk telomere lengths by TRF analysis [40–42] or the slot blot assay [43] demonstrated that the majority of invasive mammary carcinomas had shorter telomeres than adjacent, benign breast tissues. Telomere lengths in cancer cells were shorter in high-grade tumours [40] and short telomeres correlated with aneuploidy and the development of lymph node metastases [43]. Subsequently, high resolution *in situ* telomere length assessment combined with immunostaining to differentiate specific cell types [32], confirmed that significant telomere shortening is prevalent in ~70% of invasive mammary carcinomas [44]. Interestingly, ~25% of invasive breast carcinomas contain telomeres that are either similar or longer than the adjacent stromal fibroblasts [44]. Additionally, two studies have identified breast tumours displaying the ALT phenotype, a telomerase-independent telomere length maintenance mechanism characterized by remarkable telomere length heterogeneity, ranging from ultra-short to ultra-long telomeres [45, 46]. The ALT phenotype [45] has been primarily observed in sarcomas, but is relatively rare in most carcinomas [47]. Similar telomere length distributions seen in cancer cells have been observed in the preneoplastic lesions, ductal carcinoma *in situ* and lobular carcinoma *in situ* [44], although the ALT phenotype has not been reported.

Surprisingly, not only does telomere shortening occur in the majority of *in situ* and invasive breast carcinomas [48], but telomere length alterations also occur in seemingly histologically normal breast tissues. These alterations have been observed in normal terminal ductal lobular units adjacent to a tumour and even in disease-free breast tissues obtained from reduction mammoplasties [44, 49, 50]. Using Q-FISH, telomere lengths were assessed in normal lobules and normal lactiferous ducts. Strikingly, telomere shortening was observed in the majority of normal lobules, but not in normal lactiferous ducts. Notably, short telomeres were only seen in the luminal cells and not in the myoepithelial cells. This finding was confirmed in a recent study that observed telomere shortening in normal luminal and tumour cells, but not shortening in the myoepithelial or fibroblast cell populations [49]. Although interesting biologically that telomere shortening may contribute to breast cancer promoting genomic alterations, these observations most likely preclude the use of telomere length measurements, particularly bulk measurements, as a diagnostic marker in breast tissues (*e.g.* needle core biopsy specimens). Telomere length measurements in cell preparations, for example fine-needle aspirates (FNAs), may be difficult because the histological information is lost. However, telomere length measurements in breast tissues adjacent to tumours may still have clinical utility. In particular, telomere DNA content measured by the slot blot assay was observed to be decreased in benign tissues 1 cm

Table 1 Telomere length and telomerase detection methods: strengths and limitations

Method	Strengths	Limitations	References
Telomere length methods			
TRF Southern blot analysis	Widely used; provides telomere length distributions	Requires relatively large amounts of DNA (μg range); inclusion of sub-telomeric regions in the telomere length estimation; variability in interpretation; relatively laborious; cannot be used on fixed tissues	[28, 29]
Q-FISH	Allows telomere length assessment in fixed material; provides single cell resolution while maintaining tissue architecture; allows identification of telomere lengths in specific cell types	Although quantitative analysis may be performed, values are relative telomere measurements, not actual length	[30–32]
Flow-FISH	Average telomere lengths can be quantitated; provides a distribution of telomere lengths	Relatively laborious; can only be used on single cell suspensions, such as blood leucocyte samples; cannot be used on fixed tissues	[33]
Slot blot assay	Low DNA input requirement (ηg range); can used on fixed tissues	Provides mean telomere content (not length); does not provide a distribution of telomere lengths; no identification of telomere lengths from specific cell types	[34, 35]
Quantitative telomere-specific PCR (Q-PCR)	High-throughput analysis allows for assessment of large sample sets; requires low DNA input (ηg range)	Provides mean relative telomere content (not length); no distribution of telomere lengths within a particular sample; not easily performed on fixed tissues	[36, 37]
STELA	Can be used to detect telomere lengths from specific, individual chromosomes; can identity extremely short telomeres	Relatively laborious; primers for all chromosome arms have not been developed; may not detect extremely long telomeres	[38]
Telomerase detection methods			
TRAP	'Gold-standard' for telomerase activity measurements; need for only small amount of cells	Relatively laborious; hard to quantify; need for appropriate controls	[39]
Detection of transcript levels of hTERT or hTR by RT-PCR	Allows for quantitative measurement of the specific subunits; fairly high throughput; can detect slice variants	mRNA expression levels of hTERT may not directly correlate with telomerase activity due to post-transcriptional processes	
Detection of transcript levels of hTERT or hTR by *in situ* hybridization	Allows for detection and visualization of transcripts at single cell level; allows for detection in specific cell types	Detection of transcripts does not always correlate with telomerase activity; decreased sensitivity due to low abundance of hTERT may be problematic	

away from the visible tumour margin, but not to the same extent as in tissue 5 cm from the tumour margin, suggesting a possible cancer field effect [50]. The concept of cancer field effect, or field cancerization, refers to the occurrence of molecular alterations in histologically normal tissues surrounding tumours [51]. Although still preliminary, the identification of molecular alterations, such as telomere shortening, in histologically normal cells may have clinical implications for breast-sparing surgery by defining appropriate molecular tumour margins and assessing risk factors for the development of recurrent disease [52].

A newer area of investigation has focused on measuring telomere lengths in peripheral blood lymphocytes, either by TRF analysis or Q-PCR, to assess potential links between constitutive telomere length and risk of breast cancer development. However, to date, the results have been largely conflicting. When comparing telomere lengths to healthy controls, three studies have observed shorter telomeres in cancer patients [53–55], one study did not observe any significant difference [56], and two studies observed longer telomeres in cancer patients [57, 58]. Recently, a prospective cohort of postmenopausal women did not show a significant association between increased risk of developing breast cancer and telomere length in peripheral blood lymphocytes [59]. Pooley *et al.* showed that decreased mean telomere length in peripheral blood lymphocytes was associated with a significant, yet modest increased risk of developing breast cancer in a retrospective study; however, the association was not replicated in a prospectively collected cohort [60]. The authors postulated that the observed telomere shortening predominantly occurs after diagnosis, thus diminishing its potential value as a predictive risk marker for breast cancer. More research is warranted to clarify these issues.

Whereas, the above studies have investigated mean telomere length, Zheng and colleagues have focused on assessing telomere alterations on individual chromosome arms [61, 62]. Suggesting that critically short telomeres on specific chromosome arms may be an underlying mechanism for chromosome specific instability, chromosome arm-specific telomere lengths were measured by Q-FISH in short-term cultured blood lymphocytes. In the first case–control study, short telomere lengths on chromosome 9p were associated with an increased risk of breast cancer [61]. Interestingly, the *CDKN2A* gene which encodes for $p16^{INK4}$ and $p14^{ARF}$, tumour suppressor proteins that regulate the Rb and p53 pathways, is located on chromosome 9p. In premenopausal women, these findings where confirmed and extended; in addition to 9p, short telomere lengths on 15p, 15q and Xp were also associated with an increased risk of the development of breast cancer [62]. Chromosomal arm-specific telomere length analysis could be incorporated in a panel of biomarkers used for risk assessment of breast cancer.

Prostate cancer

A major characteristic of prostate cancer is prominent chromosomal instability. Prostate cancer is thought to develop from benign epithelium through high-grade prostatic intraepithelial neoplasia (PIN), the earliest precursor lesion, to invasive adenocarcinoma. Because telomere dysfunction causes chromosomal instability, Sommerfeld and colleagues studied telomere dynamics in the prostate by measuring telomere lengths in matched samples of benign prostatic hyperplasia (BPH), benign nodules composed of stromal components and epithelial cells and invasive prostatic adenocarcinomas obtained by radical prostatectomy [63]. Prostate cancer tissue telomere lengths were significantly shorter than the telomeres from cells in BPH tissues and from adjacent normal tissues. These results were confirmed [64, 65], and extended to demonstrate an association between reduced telomere lengths in prostate tumours and disease recurrence [66].

Using high-resolution *in situ* methods, telomere shortening was observed in tumour epithelial cells compared to normal prostatic epithelial cells in the vast majority of prostate tumours [32], as well as in high-grade PIN lesions [67, 68]. Within these PIN lesions, telomere shortening only occurred in the luminal epithelial cells and not in the basal epithelial cells or the surrounding stromal cells. The high prevalence of short telomeres in high-grade PIN lesions, of which only a small fraction progress to invasive carcinomas, would preclude the use of this molecular marker for prostate cancer detection. However, telomere length measurements in tumour specimens obtained at time of surgery or taken at time of biopsy do seem promising as a potential prognostic marker in prostate cancer [66, 69, 70].

To date, only one study has assessed the relationship between constitutive telomere length in peripheral blood lymphocytes and prostate cancer. Using Q-PCR, this nested case–control study showed no association between mean telomere length and risk of aggressive prostate cancer development [71]. More interestingly, recent studies have reported telomere shortening in histologically normal prostate tissues from diseased prostates. In one study, telomere lengths were assessed by Q-FISH in biopsies from a cohort of men diagnosed with high-grade PIN, but without evidence of prostate cancer [72]. The degree of telomere shortening in the surrounding stromal cells and within cells from the high-grade PIN lesions were associated with the eventual diagnosis of prostate cancer [72]. In another report, mapping of the spatial distributions of telomere DNA content, measured by the slot blot assay, revealed telomere length variations in fields of histologically normal tissues surrounding tumours in a small set of radical prostatectomies specimens [73]. Expanding on their previous work, Joshua *et al.* assessed telomere lengths topographically by Q-FISH in normal epithelium, adjacent stroma, BPH, high-grade PIN and cancer in whole mount tissue sections [74]. Here, the presence of short telomeres in different prostatic histologies correlated with telomere lengths within adjacent stromal cells, suggesting microenvironmental effects within the prostate gland, such as increased oxidative stress [74].

The lack of association between constitutive telomere length, as measured in peripheral blood lymphocytes, and increased prostate cancer risk and the existence of telomere shortening in seemingly histologically normal prostate tissues suggests measurement of telomere lengths will not be useful as a direct diagnostic marker of prostate cancer. However, it is possible that telomere length analysis could nonetheless be potentially useful. For example, in settings where there is suspicion of cancer, *i.e.* in men with persistently elevated serum prostate specific antigen (PSA) but negative biopsy results. If abnormal telomeres in the stromal or epithelial cell populations are present on the biopsy, then it may suggest an underlying defect, thus triggering a repeat biopsy.

Other cancer types

As illustrated with breast and prostate cancers, telomere lengths have been extensively studied in most cancer types [25]. In general, the majority of studies have compared tumour samples to either histologically normal, tumour-adjacent tissues or truly disease-free tissues. Although telomere alterations are found in the majority of cases, the direction of the alteration, either shortening or lengthening, appears tissue dependent and may vary within a particular tumour type. For example, a fraction of colorectal carcinomas have cancer cells with telomere lengths longer than adjacent normal cells and these cases tend to have a poor survival [75, 76]. Additionally, telomere alterations frequently occur in precursor lesions of most human epithelial cancers [77, 78]; therefore, telomere length analysis alone cannot differentiate between the presence of a precursor lesion or invasive cancer. Because most precursor lesions are not treated and will subsequently never progress to invasive carcinomas, the direct measurement of telomere length in tissue, or even in cytological preparations, is not a suitable molecular marker for the diagnosis of cancer. However, an area of investigation that may be promising is *in situ*

telomere length analysis to identify patients that are good, or poor, candidates for a particular therapy. For example, recently developed telomerase inhibitors work most effectively on cells with short telomeres; therefore, assessment of the telomere lengths, along with telomerase activity, prior to treatment would be necessary to differentiate the patients most likely to benefit from the treatment [79].

Another avenue of investigation has concentrated on telomere length measurement in peripheral blood lymphocytes which can be easily obtained. Taking advantage of the high-throughput Q-PCR assay, numerous groups have assessed mean telomere length in peripheral blood as a possible marker for the risk of development of different cancer types, including lung, bladder, oesophagus, skin, head and neck and kidney; the field has been recently reviewed by Svenson and Roos [25]. Since that comprehensive review, additional population-based investigations correlated shorter mean leucocyte telomere length with an increased risk of gastric cancer [80] and serous ovarian adenocarcinoma [81]. Conversely, longer leucocyte telomere length was associated with an increased risk of developing non-Hodgkin lymphoma [82], whereas no association between telomere length and risk of incident colorectal carcinoma was found in two recent prospective studies [83, 84]. One particularly intriguing study performed by Willeit and colleagues, analysed mean leucocyte telomere length in 787 participants free of cancer at baseline and prospectively followed for 10 years [85]. In this cohort, short telomeres were associated with subsequent cancer development independent of other cancer risk factors. Although associations could not be assessed between telomere length and each specific cancer type due to the relatively small number of cases, this inverse association is of particular interest and warrants further investigation. As with the tissue-based findings, the correlations with mean leucocyte telomere length and the risk of the development of cancer depends on the cancer type. Although, larger, prospective studies are needed, these preliminary findings suggest that telomere length analysis may provide some screening diagnostic benefit, most likely in conjunction with other molecular markers, to identify a subset of patients at risk for development of a particular cancer.

Telomerase activity as a potential diagnostic marker in cancer

Breast cancer

The development of the PCR-based TRAP assay by Kim *et al.* [39] greatly improved our ability to assess the levels of telomerase activity. Initial results demonstrated that 93% of breast cancers and only 4% of histologically normal adjacent tissues were telomerase positive [86]. Follow-up studies confirmed the presence of telomerase activity in the vast majority of invasive breast carcinomas (range 73–95%), ductal carcinoma *in situ* lesions (range 59–100%), but only in a small fraction of benign breast tissues [87–91]. However, as an ideal diagnostic marker would be available prior to surgery, the TRAP assay was slightly modified to increase sensitivity for use on FNAs [92]. In comparison to cytological preparations, >90% of breast cancers were telomerase positive, whereas, only a small fraction of benign breast lesions were positive for telomerase activity [93, 94]. Taken together, these studies suggest that detection of telomerase activity may be a useful breast cancer marker in FNAs; however, as alluded to by Mokbel and colleagues, the role of telomerase detection may only be useful as a complementary marker to a traditional cytopathological diagnosis [95].

In agreement with hTERT as the catalytic and rate-limiting telomerase component, mRNA expression levels of hTERT have been shown to roughly correlate with telomerase activity in breast cancer [96]. Several studies have measured mRNA expression levels of hTERT, and occasionally also hTR, using quantitative RT-PCR in a variety of samples. One investigation found that hTERT mRNA expression was significantly higher in breast cancer tissues compared to adjacent normal breast tissues, suggesting a possible role for the measurement of hTERT mRNA levels in breast cancer diagnosis [97]. Ultimately, a panel of markers that assesses hTERT expression levels in combination with mRNA expression profiling of other key telomere-related genes may prove beneficial for breast cancer detection [98].

More recent developments have focused on the detection of hTERT in peripheral blood from breast cancer patients, with the idea of detecting circulating tumour cells (CTC). Shen and colleagues measured mRNA levels of hTERT, survivin and mammaglobin in peripheral blood samples from breast cancer patients and healthy individuals. Individually, the sensitivity of the three markers was extremely low (33–60%), with hTERT being the highest, but the combination of the three markers increased the sensitivity to 70% and an overall specificity of 100% [99]. Another study assessed hTERT mRNA in plasma from breast cancer patients, women diagnosed with fibroadenomas and healthy controls. hTERT levels in the plasma showed a sensitivity of 50% and specificity of 90% in the ability to detect malignancy [100]. These interesting findings suggest a possible role for assaying hTERT in the detection of CTCs. In the future, telomerase measurements, in conjunction with other molecular markers, may have utility in the early diagnosis of breast cancer.

Prostate cancer

While investigating prostate telomere biology, Sommerfeld and colleagues demonstrated the presence of telomerase activity in 84% of prostatic adenocarcinomas, 12% in matched adjacent normal tissues and 0% in adjacent BPH tissues [63]. A follow-up study observed that 90% of prostate cancers were telomerase positive, whereas, normal prostate tissues were all telomerase negative [101]. As well as confirming telomerase activity in prostate cancers, Koeneman *et al.* observed telomerase in 16% of

samples of high-grade PIN [65]. Extensive evaluation of telomerase activity in prostate needle biopsies shows similar results to the observations in the radical prostatectomy tissues (reviewed in [102]). Additionally, analysis of hTR and hTERT, either by RT-PCR or by *in situ* hybridization, has shown similar trends to the earlier telomerase activity studies, whereby most invasive cancers as well as PIN lesions are positive, BPH lesions are intermediate and normal adjacent areas show low to no levels of expression (reviewed in [102]).

Telomerase activity can also be detected in bodily fluids, such as expressed prostatic secretions and urine [103–107]. Telomerase activity was detected in prostatic fluids in 83% of prostate cancer patients compared to only 11% of patients without clinical evidence of prostate cancer [103]. Attempting to improve sensitivity for detecting cancer, hTERT expression was measured in conjunction with hypermethylation of the glutathione S-transferase P1 promoter, another common molecular alteration in prostate cancer [107]. The sensitivity for this combined assay was 73%, but the specificity was only 43%. Across studies using freshly voided urine samples after prostatic massage, telomerase activity has been detected in men diagnosed with prostate cancer (range 58–100%), but also in a subset of men (range 13–30%) with BPH and no evidence of concurrent adenocarcinoma [104–106].

The most promising studies have come in the last several years. Pfitzenmaier *et al.* analysed telomerase activity in bone marrow aspirates from men with localized prostate cancer to detect disseminated prostate cancer cells [108]. Although 49% of the men had detectable telomerase activity demonstrating the feasibility of the approach, the procedure work in only half of the patients due to technical difficulties, highlighting the need for improved technology before the approach can be implemented in a clinical setting. In a different approach, Dasi and colleagues evaluated plasma hTERT mRNA levels in patients with elevated PSA levels and healthy men. Using a cut-off value (the highest value observed in the control group), the authors reported a 81% sensitivity and a 60% specificity, suggesting that hTERT mRNA levels may be able to differentiate between patients with prostate cancer and patients without evidence of disease [109]. Because free plasma DNA had been suggested to be a diagnostic marker for cancer, Altimari *et al.* assessed hTERT mRNA levels from blood samples in patients diagnosed with localized prostate cancer and determined an 80% sensitivity and 82% specificity, implying its potential use as an early diagnostic and monitoring marker for prostate cancer [110]. Finally, Fizazi and colleagues developed a method using telomerase activity to specifically detect CTCs in patients with prostate cancer. Epithelial cells from peripheral blood mononuclear cells were harvested and telomerase activity measured; CTCs were detected in 79% of patients with localized prostate cancer prior to radical prostatectomy or brachytherapy, in 79% of patients with advanced metastatic disease and in 0% of healthy patients [111]. Other capture strategies, for example the use of microfilter-based platforms to determine telomerase activity from live-captured CTCs, are currently being developed and validated [112].

Other cancer types

As with breast and prostate cancer, telomerase activity or hTERT expression is present in the vast majority of solid tumours and has proved to be a marker of malignancy [113]. However, telomerase activity or hTERT expression has been shown to be present in some normal tissues and benign conditions. Due to the size of the field, the reader is directed to other comprehensive reviews discussing the potential clinical utility of using telomerase as a diagnostic marker in cancer in general [76] or within specific tumour types [114, 115].

To provide a specific example, the detection of telomerase by non-invasive means such as analysing urine or other bodily secretions seems to be potentially useful some cancer types, in particular for bladder cancer [116, 117]. A case–control study comparing patients with confirmed bladder cancer and healthy controls analysed telomerase activity from urine samples. Using an arbitrary cut-off value, Sanchini and colleagues reported a 90% sensitivity and a 88% specificity with similar patterns observed in low-grade tumours [118]. These preliminary results seem promising, but caution must be taken because the presence of acute or chronic inflammation may affect the telomerase activity measurements. Nevertheless, the non-invasive evaluation of telomerase activity in urine may provide additional diagnostic information, independent of routine cytology and most importantly may identify low-grade tumours, which are difficult to identify by cytological examination alone.

Although measurement of telomerase activity or hTERT expression in blood may provide some diagnostic utility, concerns still remain about the specificity of the telomerase activity measurements because activated lymphocytes display telomerase activity and any lymphocyte contamination is a possible confounder. Although, hTR and hTERT have been analysed at the RNA transcript level, it is still a major limitation that there are no reliable hTERT antibodies for use in immunohistochemistry. Although several antibodies are claimed to be specific for hTERT, none have been adequately validated in tissues. For example, it was even shown that a widely used antibody recognized nucleolin, not telomerase [119]. Telomerase antibodies are still being generated and evaluated; however, the detection in tissue has been problematic, likely due to the relatively low abundance of the telomerase protein. Newer antibody amplification techniques may prove beneficial and establishment of immunohistochemical protocols would open new research avenues for cancer diagnostics.

Interestingly, recent large, high-resolution analyses of somatic DNA copy-number alterations revealed that the *TERT* gene is located in one of the most significant focal amplifications in lung adenocarcinoma [120] and across multiple other cancer types [121]. Likewise, genomic amplification of the *TERC* gene, which codes for hTR, has been associated with the development of invasive carcinomas, for example in lung cancer [122], but most notably has been associated in the development of cervical cancer and may aid in the diagnosis of low-grade lesions when combined with cytology [123–125]. Additionally, studies stemming from the large-scale genome-wide association studies efforts have

discovered sequence variants in the TERT-CLPTM1L locus on chromosome 5p15.33 that associated with increased risk of cancer development [126–129]. Although these associations are modest, this TERT polymorphism has been statistically associated with increased risk of basal cell carcinoma and cancers of the lung, bladder, prostate, cervix and pancreas. However, it must be noted, that this association was not confirmed by another group analysing the polymorphism and risk of breast cancer, colorectal cancer and melanoma [130]. Nevertheless, these genome-wide association studies still may provide useful information implicating variation in the *TERT* gene as a cancer risk factor [131]. Ultimately, a unique single nucleotide polymorphism or a panel of single nucleotide polymorphisms, may provide useful diagnostic information and may identify a subset of people at an increased risk for the development of cancer.

Future directions

An exciting new area of ongoing research focuses on the assessment of telomere dysfunction, rather than telomere length alone, as a potential marker in cancer. In general, the telomere is 'capped', or functional, when the Shelterin complex is bound to the telomere, protecting the end of the chromosome from being recognized as a double-strand break. However, when the telomere is no longer protected, then the telomere is considered 'uncapped', or dysfunctional [4]. At this point, the telomere no longer inhibits the DNA damage response pathway and is prone to telomere fusions *via* the non-homologous end joining DNA repair pathway [7]. Such chromosome fusions can initiate genomic instability because the resulting dicentric chromosomes can be pulled to opposite poles during mitosis, eventually breaking, thus creating a cycle of breakage, fusion and bridging. This carcinogenesis-promoting genomic instability occurs when telomeres become critically short in the presence of abrogated tumour-suppressive checkpoint pathways, such as p53 and pRb [132]. Consistent with the notion that critical telomere shortening causes telomere dysfunction, Hemann *et al.* demonstrated that the shortest telomere within a cellular population can generate telomere dysfunction [133]. More recently, a large-scale genome sequencing study revealed that pancreatic cancer acquires genomic rearrangements consistent with telomere dysfunction [134]. Additionally, telomere dysfunction resulting from telomere shortening can induce tetraploidization that drives tumorigenesis [135].

Although telomere shortening can eventually lead to telomere dysfunction, recent investigations have shown that there are multiple mechanisms that may lead to telomere dysfunction. For example, alterations in TRF2, a major component of the Shelterin complex, lead to uncapping of the telomere even in the presence of adequate telomeric repeat sequences [136, 137]. Another investigation demonstrated that telomere dysfunction can increase telomeric homologous recombination in cancer cells, as monitored by telomere sister chromatid exchanges, even in the presence of telomerase [138]. Additionally, recent observations have demonstrated the critical role of the Shelterin component Rap1 for repressing homology-directed repair at telomeres [139]. New investigations on the cellular consequences of short telomeres have highlighted the importance of the induction of the DNA damage response pathway. Telomere-dysfunction induced foci can be visualized by the accumulation of γH2AX at the telomere [140]. Ultimately, it is hoped that further insights into the induction of the DNA damage response at the telomere will provide future avenues of study that may provide translational utility.

Although previous investigations have assessed telomere shortening in cancer cells, these length measurements have been considered a proxy for telomere dysfunction. The schematic in Figure 1 highlights the finding that the majority of tumours have telomere length alterations, either shortening or lengthening, that can eventually lead to telomere dysfunction. Although telomere length changes *per se* are unlikely to be useful diagnostic biomarkers, identification of the fundamental underlying molecular changes that cause telomere destabilization may unmask new markers that can aid in diagnosis of cancer.

Conclusions

Telomeres and telomerase have been focal points of cancer research for several decades. The dynamic interplay between telomeres and telomerase is critical in the development and progression of human cancer. The diagnostic utility of measurements of telomere length, or content, in solid tumours has been assessed. Early studies highlighted significant shortening in telomere lengths in cancer cells compared with normal adjacent cells from a variety of tissue types. However, more refined techniques, such as Q-FISH, have revealed more complex telomere phenotypes, including the presence of telomere alterations in premalignant and even normal-appearing cells, thus impacting the cancer specificity of telomere length changes. In addition, cancer cell telomere lengths vary considerably among different cancers. In some tumour types (*e.g.* breast cancer), there can be extreme heterogeneity within a particular cancer type and even within an individual tumour. Therefore, the use of telomere length measurements alone may not be suitable as a cancer diagnostic marker. However, telomere length measurements in tumour tissue, either from surgical specimens or specimens taken at the time of biopsy, or from other bodily fluids may serve as a molecular marker for risk assessment, prediction for response to therapy (*e.g.* setting of telomerase inhibitors) or prognosis.

Significant differences in telomerase activity and expression levels of hTERT have been observed between cancerous and benign tissues and assessed for possible diagnostic utility. However, it has been shown that telomerase may also be present in a small fraction of some benign lesions (*e.g.* fibroadenomas of the breast), pre-invasive lesions (*e.g.* high-grade PIN), as well as inflammatory cells. Therefore, the diagnostic use of the detection

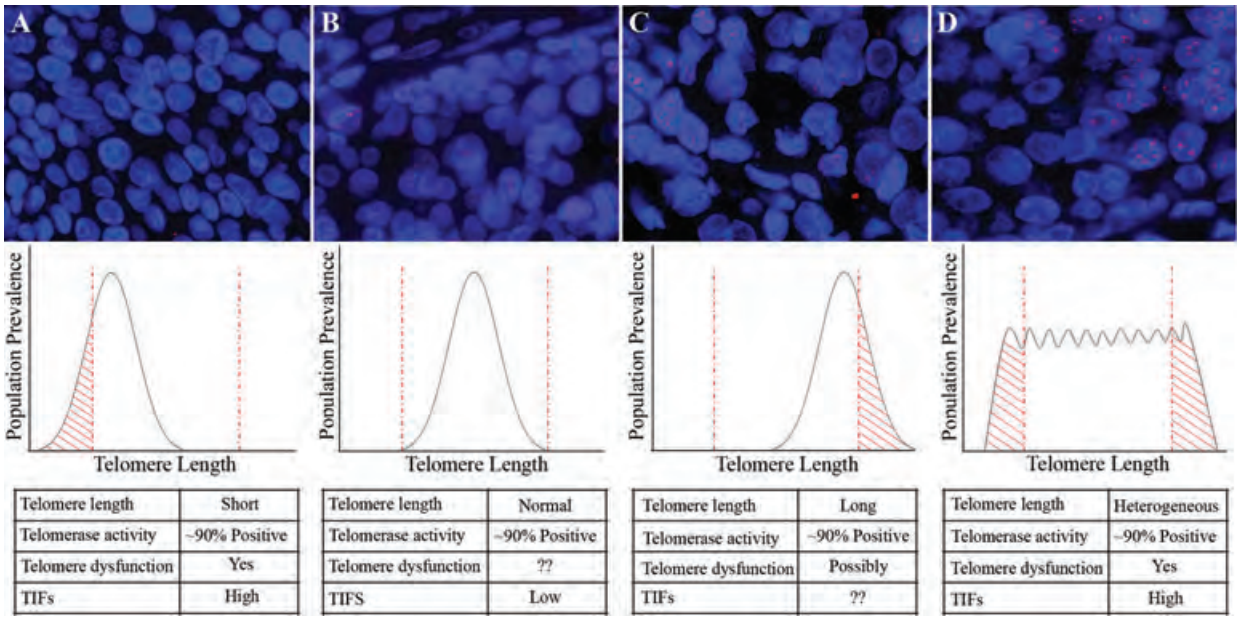

Fig. 1 Model depicting the possible relationships between telomere lengths, telomerase activity and telomere dysfunction in human carcinomas. Telomere length analysis by FISH from representative examples of tumours displaying (**A**) extremely diminished telomere signals in cancer cells, (**B**) comparable telomere intensities in cancer cells and benign stromal cells, (**C**) extremely bright telomere signals in cancer cells compared to benign stromal cells and (**D**) heterogeneous cancer cell telomere lengths varying from extremely short to relatively long. For the images (original magnification ×400), the DNA is stained with DAPI (blue) and telomeric DNA is stained with a Cy3-labeled telomere-specific peptide nucleic acid probe (red). Below each panel is a proposed model depicting the telomere length distributions in each tumour and the relationship to telomere dysfunction. The critical threshold levels for telomere function for critically short and abnormally long telomeres are shown (dashed red lines). Although ~90% of tumours display telomerase activity, the cancer cell telomere lengths may vary drastically. Thus, ongoing investigations into the cause of telomere dysfunction may unravel new molecular markers with potential translational utility.

of telomerase activity in tissues seems limited except in specific circumstances, such as detection of CTCs or in urine to detect bladder cancer.

In summary, although initial expectations for the use of telomere shortening or telomerase activity as highly specific markers of cancer have since been tempered, there are many avenues for research being investigated that may provide new molecular markers related to telomere biology. In particular, the area of telomere dysfunction, rather than telomere length alone, may yield new insights not only into the pathogenesis of a particular cancer, but could also have major implications in the diagnosis of cancer.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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