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Award Number: W81XWH-05-1-0273

TITLE: Evaluation of Genomic Instability as an Early Event in the
Progression of Breast Cancer

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REPORT DATE: April 2008

TYPE OF REPORT: Annual Summary

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

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1. REPORT DATE (DD-MM-YYYY) 30-04-2008		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 1 APR 2005 - 31 MAR 2008	
4. TITLE AND SUBTITLE Evaluation of Genomic Instability as an Early Event in the Progression of Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0273	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Christopher M. Heaphy, Ph.D. Jeffrey K. Griffith, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of New Mexico Health Sciences Center 915 Camino de Salud, NE Albuquerque, New Mexico 87131-5221				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We have shown in two independent retrospective studies that loss of telomere DNA content (TC) has potential value in predicting clinical outcome in breast cancer. However, an alternative marker for TC, which could be assessed in samples with small numbers of cells, such as fine needle aspirates, with commonly used methods is desirable. The aim of this study was to demonstrate that measurement of allelic imbalance (AI), which could be easily adapted to the clinical laboratory setting, could serve as a surrogate for TC, discriminating between women in need of more aggressive treatment and those for whom aggressive protocols are unnecessary. The candidate has developed a robust assay to determine the extent of AI that discriminates between normal and tumor specimens with 67% sensitivity and 99% specificity. Additionally, the candidate has shown that increased AI and altered TC are present in both tumors and surrounding histologically normal breast tissues at distances at least one centimeter from the visible tumor margins and decrease as a function of distance. In addition to evaluating a potential biomarker of breast cancer progression, the proposed investigation has provided the candidate opportunities to interact with pathologists and oncologists to learn normal and abnormal breast morphology, the strengths and limitations of currently used breast cancer biomarkers and the scientific rationale for ongoing clinical trials. To date, all tasks, as outlined in the Statement of Work, have been completed or partially completed.					
15. SUBJECT TERMS Allelic Imbalance, Genomic Instability, Human Breast Cancer, PCR					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT unlimited	18. NUMBER OF PAGES 56	19a. NAME OF RESPONSIBLE PERSON Jeffrey K. Griffith
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			19b. TELEPHONE NUMBER (include area code) (505) 272-8432

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I. INTRODUCTION

Our laboratory has shown in two independent retrospective studies that loss of telomere DNA content (TC), a surrogate for telomere length, has potential value in predicting clinical outcome in breast cancer. While TC appears to provide a sensitive predictor of disease-free survival in women with breast cancer, an alternative marker for TC, which could be assessed in samples with small numbers of cells, such as fine needle aspirates, with commonly used methods, such as polymerase chain reaction (PCR), is desirable. The aim of this study was to demonstrate that measurement of allelic imbalance (AI), which could be easily adapted to the clinical laboratory setting, can serve as a surrogate for TC, discriminating between women in need of more aggressive treatment and those for whom aggressive protocols are unnecessary. In addition to evaluating a potential biomarker of breast cancer progression, the proposed investigation provided the candidate 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, have been completed or partially completed.

Hypothesis and Rationale

Our preliminary results suggested that the extent of AI may have prognostic value in breast cancer. Consistent with this notion, Kronenwett and colleagues have shown that the degree of genomic instability allows additional classifying of the known aneuploid, diploid, and tetraploid categories of primary breast adenocarcinomas into low and high malignant subtypes. Therefore, *we hypothesized that measuring the extent of AI at diverse microsatellite loci provides a global assessment of overall genomic instability in a tumor and its surrounding microenvironment and has value in predicting breast cancer progression.* To test this hypothesis we assessed the potential prognostic value of AI in human breast tumor samples. Additionally, we studied AI in co-existing histologically normal (CHN) breast tissue and in stromal and epithelial cell populations. This hypothesis was evaluated through three specific aims.

- **Specific Aim #1:** *To assess the potential use of allelic imbalance in predicting disease-free survival by conducting a retrospective study on node negative breast tumors.*
- **Specific Aim #2:** *To assess the extent of allelic imbalance as a function of distance from tumor margins in breast tumors and co-existing histologically normal breast tissue, to determine if stromal and epithelial cells display different patterns of allelic imbalance, and to identify molecular signatures associated with the extent of allelic imbalance in stromal and epithelial cells.*
- **Specific Aim #3:** *To compare the extent of allelic imbalance to pathological grading in invasive breast tumors by conducting a prospective study on breast tumors.*

II. KEY ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

During the three years of this training grant, I have accomplished the following:

- Demonstrated that altered telomere DNA content (Figure 1, Appendix A) and unbalanced allelic loci (Figure 3, Appendix A) are present in both tumors and surrounding CHN breast tissues at distances at least one centimeter from the visible tumor margins and decrease as a function of distance. Additionally, unbalanced loci were conserved between the surrounding breast tissues and the tumors, implying cellular clonal evolution (Figure 4, Appendix A).
- Determined that TC in a cohort of breast cancer tissues (N=77) was associated with tumor size, nodal involvement, TNM (Tumor-Nodes-Metastasis) stage, 5-year overall survival and 5-year disease-free survival (Figure 2, Appendix B). A multivariable Cox model demonstrated that TC predicts breast cancer-free survival interval independent of age at diagnosis and TNM stage (Table 5, Appendix B). These findings were further validated by analyzing TC in 530 tumor specimens obtained from the New Mexico subset of the NCI/SEER Health, Eating, Activity and Lifestyle (HEAL) prospective, population-based cohort. In this larger study, TC predicted overall survival interval (Figure 1, Appendix C). Additionally, TC predicted breast cancer-free survival interval in this group (Figure 2, Appendix C) independent of TNM stage, p53 status and estrogen receptor (ER) status (Table 2, Appendix C).
- Measured AI in normal and tumor specimens from varying organs and determined that the AI method, developed by the candidate, is able to discriminate between normal and tumor specimens with 67% sensitivity and 99% specificity (Figure 3, Appendix D).
- Assessed TC (Figure 1, Appendix E) and AI (Figure 2, Appendix E) in 54 histologically normal tissues, 10 atypical ductal hyperplasias (ADH), 122 *in situ* ductal carcinomas (DCIS), and 348 Stage I, 144 Stage IIA, 39 Stage IIB and 4 Stage IIIA invasive carcinomas. The results demonstrated that genomic instability (*i.e.* increased AI and alterations in TC) increases in ADH and plateaus in DCIS without further increase in the invasive carcinomas, supporting the notion that invasive carcinomas evolve from or in parallel with DCIS (Table 2, Appendix E).
- Demonstrated consistent differences in gene expression by conducting microarray analyses in 5 patient-matched, tumor-adjacent, histologically normal tissues obtained from sites 1 cm (TAHN-1) and 5 cm (TAHN-5) from the visible tumor margin. Thirty-one transcripts of known genes were identified to be ≥ 2 fold over-expressed in TAHN-1 compared to TAHN-5 tissues, including collagens alpha 1(I), 1(III) and 2 (I) (Table 1, Appendix F). This is particularly provocative, as increased collagen synthesis is indicative of reactive stroma, which may act as a supportive agent in tumorigenesis. Five transcripts were identified to be ≥ 2 fold under-expressed in the TAHN-1 compared to

TAHN-5 tissues (Table 2, Appendix F) and 23 transcripts were identified to be ≥ 4 fold over-expressed in both TAHN-1 and TAHN-5 tissues (Table 3, Appendix F).

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the activation of this award, the Principle Investigator (PI) has been provided the opportunity to work and interact with oncologists, surgeons, pathologists and other scientists who all specialize in breast cancer. The PI has attended journal clubs, specialized departmental and Cancer Center seminars and has been an active participant in the Breast Multidisciplinary Working Group. The PI's research was overseen by his dissertation committee, a group comprised of three Ph.D. scientists with interests in breast cancer, and one M.D. who specializes in breast cancer pathology.

In addition, the PI helped instruct four upper-level Biochemistry courses: (i) Introductory Biochemistry, (ii) Biochemical Methods Laboratory, (iii) Intensive Biochemistry I and (iv) Intensive Biochemistry II: Intermediary Metabolism. The PI was also a teaching assistant for a graduate level Cancer Biology course and the Genetics and Neoplasia block of the Medical School Curriculum. The PI plans on continuing his cancer research in an academic setting; thus, these valuable teaching experiences will provide him with the necessary teaching skills to further his career.

IIc. PERFORMANCE ACCOMPLISHMENTS

Experimental Milestones

Specific Aim 1 (4 tasks)

- | | | |
|--------|--------------|---|
| Task 1 | Month 1-12 | Completed |
| | | <ul style="list-style-type: none">○ Identify and procure archival specimens from the New Mexico Tumor Registry (NMTR) at the University of New Mexico School of Medicine based on patient recurrence status.- In year one, 184 node negative breast tumors were procured from NMTR.- In year two, an additional set of 312 node negative breast tumors were obtained. |
| Task 2 | Months 12-14 | Completed |
| | | <ul style="list-style-type: none">○ Isolate DNA from the paraffin-embedded breast tumors.- In year one, DNA was isolated from all 184 collected specimens.- In year two, DNA was isolated from the additional set of 312 cases. |
| Task 3 | Months 14-24 | Completed |
| | | <ul style="list-style-type: none">○ Measure AI in the paraffin-embedded breast tumors.- In year one, AI was successfully determined in 172 of the 184 collected samples.- In year two, AI was successfully determined in 280 of the 312 additionally collected specimens. |
| Task 4 | Months 24-30 | Completed |
| | | <ul style="list-style-type: none">○ Analyze the correlation between the AI and patient recurrence status. |

- In years two and three, the analysis of the correlation between the extent of AI and patient recurrence status was completed.

Specific Aim 2 (7 tasks)

- Task 1 Months 1-30 **Completed**
- Prospectively, collect mastectomies and CHN breast tissues (1cm and 5cm from visible tumor margins).
 - In year one, 17 cases (tumor, 1cm, 5cm tissues) were prospectively collected.
 - In year two, an additional 11 cases were prospectively collected.
 - In year three, an additional 13 cases were prospectively collected.
- Task 2 Months 6-32 **Completed**
- Assess the pathological stage and grade by immunohistochemical techniques of the collected tissue samples with the assistance of Dr. Nancy Joste, Chief of Surgical Pathology.
 - In year one, the pathological stage and grade were assessed on 12 of the initial 17 cases.
 - In year two, the pathological stage and grade were assessed on the other 5 cases collected in year one and on 7 of the 11 cases collected in year two.
 - In year three, the pathological stage and grade were assessed on the 4 remaining cases collected in year two and on all 13 cases collected in year three.
- Task 3 Months 6-32 **Completed**
- Isolate genomic DNA from tumor and CHN tissue specimens and determine extent of AI as a function of distance from tumor margin.
 - In year one, isolation of genomic DNA and determination of the extent of AI was determined in 12 of the 17 collected cases (Figure 3, Appendix A).
 - In year two, isolation of genomic DNA and determination of the extent of AI was determined in the remaining 5 cases collected in year one and in 5 of the 11 cases collected in year two.
 - In year three, isolation of genomic DNA and determination of the extent of AI was determined in the remaining 6 cases collected in year one and in 5 of the cases collected in year three.
- Task 4 Months 6-32 **Partially Completed**
- Isolate stromal and epithelial cell populations from selected CHN tissue specimens by LCM.
 - Due to limitations in RNA isolation from normal breast tissues, isolation of cell populations by LCM was not feasible. Therefore, RNA was isolated from “bulk” histologically normal breast tissues.
- Task 5 Months 6-32 **Partially Completed**
- Extract RNA from isolated cell populations.
 - In year two, RNA was extracted from “bulk” (*i.e.* no isolation of specific cellular populations) tissues.

- Task 6 Months 9-32 **Partially Completed**
- Measure extent of AI in epithelial and stromal cell populations.
 - In year two, the extent of AI was determined in the “bulk” tissues.

- Task 7 Months 12-32 **Partially Completed**
- Perform expression analysis using stromal and epithelial cell RNA from CHN tissues by microarray hybridization. Determine molecular signatures as a function of distance from the visible tumor margins using cluster analysis.
 - In year two, due to limitations in RNA isolation from normal breast tissues, microarray hybridization experiments were performed on “bulk” breast tissues 1cm from tumor margin (N=5), breast tissues 5cm from tumor margin (N=5), and compared to 10 pooled RNAs from normal breast tissues.
 - In years two and three, we identified 31 transcripts of known genes were identified to be ≥ 2 fold over-expressed in TAHN-1 compared to TAHN-5 tissues, including collagens alpha 1(I), 1(III) and 2 (I) (Table 1, Appendix F). This is particularly provocative, as increased collagen synthesis is indicative of reactive stroma, which may act as a supportive agent in tumorigenesis. Five transcripts were identified to be ≥ 2 fold under -expressed in the TAHN-1 compared to TAHN-5 tissues (Table 2, Appendix F) and 23 transcripts were identified to be ≥ 4 fold over-expressed in both TAHN-1 and TAHN-5 tissues (Table 3, Appendix F)
 - In year three, we validated the mRNA levels of three over-expressed genes selected for analysis, which included type III collagen, alpha-1 subunit (COL3A1), type I collagen, alpha-1 subunit (COL1A1), and early growth response 1 (EGR1) by quantitative reverse transcriptase PCR (qRT-PCR) (Appendix G). These results are currently being validated with immunohistochemistry (IHC), thus showing which cellular populations (epithelial or stromal) are over expressing the protein of interest.

Specific Aim 3 (5 tasks)

- Task 1 Months 1-12 **Completed**
- Procure fresh mastectomy specimens from the University of New Mexico Cancer Research and Treatment Center (UNM-CRTC). These may be same samples collected in aim 2.
 - In year one, 17 cases (tumor, 1cm, 5cm tissues) were collected.
 - In year two, an additional 11 cases were prospectively collected.
 - In year two, due to limitations in the number of prospectively collected specimens, a cohort of retrospectively collected breast specimens, consisting of 52 reduction mammoplasty samples, 76 histologically normal tumor adjacent tissues, 34 benign breast disease cases, and 779 breast tumors (Stage 0-IV) was collected.
 - In year three, an addition 13 cases were prospectively collected.

- Task 2 Months 6-18 **Completed**
- Assess the pathological stage and grade by immunohistochemical techniques of the collected tissue samples with the assistance of Dr. Nancy Joste, Chief of Surgical Pathology.

- In year one, the pathological stage and grade were assessed on 12 of the initial 17 prospectively collected cases.
- In year two, the pathological stage and grade were assessed on the other 5 cases prospectively collected in year one and on 7 of the 11 cases prospectively collected in year two.
- In year three, the pathological stage and grade were assessed on the 4 remaining cases prospectively collected in year two and on all 13 cases prospectively collected in year three.

Task 3 Months 6-18 Completed

- o Isolate DNA from breast tumors and measure AI.
- In year one, isolation of genomic DNA and determination of the extent of AI was determined in 12 of the 17 prospectively collected cases (Figure 3, Appendix A).
- In year two, isolation of genomic DNA and determination of the extent of AI was determined in the remaining 5 cases prospectively collected in year one and in 5 of the 11 cases prospectively collected in year two.
- In year two, AI was determined in the remaining 5 cases prospectively collected in year one and in 5 of the 11 cases prospectively collected in year two.
- In year two, AI was determined in the retrospectively collected cohort of breast specimens, consisting of 54 reduction mammoplasty samples, 10 atypical ductal hyperplasias, histologically normal tumor adjacent tissues, 34 benign breast disease cases, and 657 breast tumors (Stage 0-IV) (Figure 2, Appendix E).
- In year three, AI was determined in the remaining 6 cases prospectively collected in year one and in 5 of the cases prospectively collected in year three.

Task 4 Months 24-30 Completed

- o Analyze the correlation between the AI and pathological stage.
- In year two, the correlation between AI and pathological stage was assessed. The extent of AI increased in ADH (compared to normal, disease-free tissues) and plateaus in DCIS without further increase in the invasive carcinomas (Figure 2, Appendix E).

Task 5 Months 18-36 Completed

- o Prepare and submit manuscripts.
- Five manuscripts have been published (Appendices A, B, C, D, E).

Education and Training Milestones (6 tasks)

Task 1 Months 1-6 Completed

- o Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Nancy Joste, Chief of Surgical Pathology.

Task 2 Months 6-12 Completed

- o Learn staining procedures and significance of histological markers commonly used in breast cancer under the guidance of Dr. Nancy Joste, Chief of Surgical Pathology.

- Task 3 Months 1-24 **Completed**
- Interact with oncologists (Dr. Aroop Mangalik and Dr. Melanie Royce) in the University of New Mexico Hospital to gain perspective on breast cancer research.
- Task 4 Months 1-36 **Completed**
- Attend tumor board meetings and monthly Cancer Research and Treatment Center meetings to gain understanding of current treatments for breast cancer and ongoing clinical trials.
 - Due to HIPAA regulations, the candidate was not allowed to attend tumor board meetings. However, the candidate still attended specialized departmental and Cancer Center seminars and is an active participant in the Breast Multidisciplinary Working Group through the University of New Mexico Cancer Center.
- Task 5 Months 12-18 **Completed**
- Attend the University of New Mexico School of Medicine Undergraduate Medical Education Curriculum Neoplasia block.
 - In year three, the candidate was a teaching assistant for the Genetics and Neoplasia block of the Undergraduate Medical Education Curriculum. The candidate attended lectures and served as a tutor for the small group problem-based learning section of the course.
- Task 6 Months 12-36 **Completed**
- Present ongoing work at local and national meetings.
 - In year one, the candidate presented work at three national meetings, two poster presentations and an oral presentation, and was a co-author on another poster presentation.
 - In year two, the candidate presented a poster at a national meeting.
 - In year three, the candidate presented a poster based on the work supported by this grant at a national meeting (Appendix H), and was co-author on five other poster presentations at national meetings.

III. REPORTABLE OUTCOMES

- On March 11, 2008, the PI successfully defended (*with Distinction*) his dissertation.
- The PI started a Postdoctoral Fellowship in Dr. Alan Meeker's Laboratory in the Department of Pathology at The Johns Hopkins University School of Medicine. The PI is continuing in the breast cancer research field.

Publications:

C.M. Heaphy, M.Bisoffi, C.A. Fordyce, C.M. Haaland, W.C. Hines, N.E. Joste and J.K. Griffith. Telomere DNA content and allelic imbalance demonstrate field cancerization in

histologically normal tissue adjacent to breast tumors. *International Journal of Cancer*, 119:108-116, 2006. (Appendix A)

C.A. Fordyce,* **C.M. Heaphy***, M. Bisoffi, J.L. Wyaco, N.E. Joste, A. Mangalik, K. Baumgartner, R. Baumgartner, W.C. Hunt and J.K. Griffith. Telomere content correlates with stage and prognosis in breast cancer. *Breast Cancer Research and Treatment*, 99:193-202, 2006.

**Authors contributed equally to this study (Appendix B)*

C.M. Heaphy, K.B. Baumgartner, M. Bisoffi, R.N. Baumgartner and J.K. Griffith. Telomere DNA content predicts breast cancer-free survival interval. *Clinical Cancer Research*, 13:7037-7043, 2007. (Appendix C)

C.M. Heaphy, W.C. Hines, K.S. Butler, C.M. Haaland, G. Heywood, E.G. Fischer, M. Bisoffi and J.K. Griffith. Measurement of genome-wide allelic imbalance in human tissue using a multiplex PCR system. *Journal of Molecular Diagnostics*, 9:266-271, 2007. (Appendix D)

C.M. Heaphy, M. Bisoffi, N.E. Joste, K.B. Baumgartner, R.N. Baumgartner and J.K. Griffith. Genomic Instability Demonstrates Similarity between DCIS and Invasive Carcinomas. *Breast Cancer Research and Treatment*, Epub:Sep 11, 2008. PMID: 18785004 (Appendix E)

Published Abstracts:

C.M. Heaphy, M. Bisoffi, C.A. Fordyce, A. Mangalik and J.K. Griffith (2005) Telomere DNA content and allelic imbalance in histologically normal tissue adjacent to breast tumors. Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP). Philadelphia, PA.

C.M. Heaphy, M. Bisoffi, C.A. Fordyce, C.M. Haaland-Pullus, W.C. Hines, N.E. Joste and J.K. Griffith (2005) Telomere DNA Content and Allelic Imbalance Predict Disease-free Survival and Define Field Cancerization in Histologically Normal Tissue Adjacent to Breast Tumors. San Antonio Breast Cancer Symposium. San Antonio, TX.

C.M. Heaphy, C.A. Fordyce, M. Bisoffi, J.L. Wyaco, N.E. Joste, A. Mangalik, K. Baumgartner, R. Baumgartner, W.C. Hunt and J.K. Griffith (2006) Telomere content correlates with stage and prognosis in invasive breast cancer. 1st Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE). Washington, D.C.

C.M. Heaphy, K.B. Baumgartner, M. Bisoffi, R.N. Baumgartner and J.K. Griffith (2007) Telomere DNA Content Predicts Overall and Breast Cancer-free Survival Intervals. AACR Special Meeting: Advances in Breast Cancer Research. San Diego, CA. (Appendix H)

IV. CONCLUSIONS

All tasks, as outlined in the Statement of Work, have been completed or partially completed. Since the initiation of this training grant three years ago, five manuscripts have been published. The PI defended his dissertation on March 11, 2008 and passed *with Distinction*. The applicant accepted a Postdoctoral Fellowship in breast cancer research at The Johns Hopkins University School of Medicine and started in August of 2008.

Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors

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Cancer arises from an accumulation of mutations that promote the selection of cells with progressively malignant phenotypes. Previous studies have shown that genomic instability, a hallmark of cancer cells, is a driving force in this process. In the present study, two markers of genomic instability, telomere DNA content and allelic imbalance, were examined in two independent cohorts of mammary carcinomas. Altered telomeres and unbalanced allelic loci were present in both tumors and surrounding histologically normal tissues at distances at least 1 cm from the visible tumor margins. Although the extent of these genetic changes decreases as a function of the distance from the visible tumor margin, unbalanced loci are conserved between the surrounding tissues and the tumors, implying cellular clonal evolution. Our results are in agreement with the concepts of “field cancerization” and “cancer field effect,” concepts that were previously introduced to describe areas within tissues consisting of histologically normal, yet genetically aberrant, cells that represent fertile grounds for tumorigenesis. The finding that genomic instability occurs in fields of histologically normal tissues surrounding the tumor is of clinical importance, as it has implications for the definition of appropriate tumor margins and the assessment of recurrence risk factors in the context of breast-sparing surgery.

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Key words: telomere loss; allelic imbalance; genomic instability; cancer field effect; breast cancer

Genomic instability is an important factor in the progression of human cancers.^{1–4} One mechanism that underlies genomic instability is loss of telomere function.^{5–7} Telomeres are nucleoprotein complexes located at the ends of eukaryotic chromosomes. Telomeres in human somatic cells are composed of 1,000 to 2,000 tandemly repeated copies of the hexanucleotide DNA sequence, TTAGGG.⁸ Numerous telomere binding proteins are associated with these repeat regions and are important for telomere maintenance.^{9,10} Telomeres stabilize chromosome ends and prevent them from being recognized by the cell as DNA double-strand breaks, thereby preventing degradation and recombination.¹¹ However, telomeres can be critically shortened, and thereby become dysfunctional, by several mechanisms, including incomplete replication of the lagging strand during DNA synthesis,¹² loss or alterations of telomere-binding proteins involved in telomere maintenance,¹³ and oxidative stress leading to DNA damage.¹⁴ Alternatively, telomere loss may be compensated for by recombination^{15,16} or, as seen in the majority of human cancers, by the enzyme telomerase.^{17,18}

Telomeres in tumors are frequently shorter than in the matched adjacent normal tissues, presumably reflecting their extensive replicative histories.^{19–21} The cause-and-effect relation between dysfunctional telomeres and genomic instability implies that shortened telomeres are also associated with altered gene expression. The latter is a primary source of phenotypic variability, which in turn drives the development of cell clones displaying progressively malignant traits, such as the potential for invasion and metastasis.²² In agreement with this sequence of events, we and others have shown that telomere length, or its surrogate, telomere DNA content (TC), predicts the course of disease in several different malignancies, including leukemias,²³ non-small cell lung cancers,²⁴ neuroblastomas,²⁵ prostatic adenocarcinomas,^{26–28} and breast carcinomas.^{29,30}

Recently, Meeker and colleagues observed that telomere length abnormalities are early and frequent events in the malignant trans-

formation of several types of cancer, including breast.^{27,31,32} In addition, telomere attrition and other measures of genomic instability, such as allelic imbalance (AI) and loss of heterozygosity, demonstrate that genomic instability occurs within atypical breast hyperplasias,^{33–35} histologically normal tissue proximal to breast tumors,^{36–42} and, in some instances, breast tissue from women with benign breast disease.⁴³ Loss of heterozygosity and AI have also been found in the stromal compartment of cancer-associated breast tissues.^{41,44} In addition, our own recent results identified fields of telomerase-positive cells within histologically normal tissues adjacent to breast tumors that could represent areas of pre-malignant cell populations.⁴⁵ Similarly, we have recently reported on the occurrence of telomere attrition in histologically normal prostatic tissue proximal to prostate adenocarcinomas.²⁸ These data imply that there is a reservoir of genetically unstable cell clones within histologically normal breast and prostate tissues that may represent fertile ground for tumor development. The origin and extent of this reservoir are presently undefined. However, the existence of fields of genetically altered cells, appearing histologically normal and disease-free, is consistent with the hypothesis that genomic instability arises early in breast tumorigenesis.

The primary goal of the present study was to define the extent and spatial distribution of genomic instability in histologically normal tissues surrounding breast tumors. A secondary goal was to investigate the relationship between genetic alterations in tumors and matched tumor-adjacent histologically normal (TA-HN) tissues. Towards these ends, two independent, yet conceptually linked markers of genomic instability, TC and AI, were investigated in two independent cohorts of breast tumors and their matched TA-HN tissues. One cohort represented a controlled study with tumors and matched TA-HN tissues excised at sites 1 and 5 cm from the tumor margins. The second cohort consisted of archival tumor specimens and matched TA-HN tissues excised at unknown distances from the tumor margin. Our results show that breast tumors reflect the properties of the matched TA-HN breast tissues, including the conservation of unbalanced alleles. Furthermore, our results support the hypothesis that fields of histologically normal, but genetically unstable cells provide a fertile ground for tumorigenic events in breast tissues.

Materials and methods

Breast tissue samples

Four independent cohorts of human breast tissues were used in this study. The characteristics of each of these cohorts are sum-

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Grant sponsor: DOD BCRP grants; Grant numbers: DAMD-17-01-1-0572, DAMD 17-00-1-0370, DAMD17-02-1-0514; Grant sponsor: NIH grants; Grant numbers: R25 GM60201, T34 GM08751.

Received 17 August 2005; Accepted after revision 9 December 2005

DOI 10.1002/ijc.21815

Published online 31 January 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – CLINICAL CHARACTERISTICS OF TUMOR COHORTS

Cohort	N	Age at Dx ¹			Dx ¹			Size ²		Node ³		TNM Stage						
		Range	Median	Mean	IDC	LC	DCIS	S	L	N	P	n/av	I	IIA	IIB	IIIA	IIIB	IV
1	12	26–61	53	49	10	1	1	n/av		2	10	2	0	3	2	2	3	0
2	38	35–75	48	50	36	2	0	4	32	7	29	2	2	5	14	11	0	2
3	48	31–89	54	56	44	4	0	8	40	19	29	0	11	13	15	8	1	0
4 (Normal)	20	15–48	30	29	n/a	n/a	n/a	n/a		n/a		n/a						

TNM, Tumor-Nodes-Distant Metastasis; n/a, not applicable; n/av, not available.

¹Dx, Diagnosis of invasive ductal carcinoma (IDC), lobular carcinoma (LC), ductal carcinoma in situ (DCIS). –²S = small (≤ 2 cm), L = large (> 2 cm). –³N = negative, P = positive.

marized in Table I. The first cohort consisted of 12 full mastectomy cases obtained consecutively from the University of New Mexico (UNM) Hospital Surgical Pathology Laboratory in 2003 and 2004. Approximately 500 mg of tissue was excised from the tumors and sites 1 and 5 cm from the visible tumor margins. After resection, the tissues were immediately frozen in liquid nitrogen. Sections (10–12 μ m) were prepared and stained with hematoxylin and eosin by the Human Tissue Repository Service of the UNM Department of Pathology. The sections were examined microscopically to define their histological status. In addition, serial sections of the breast tumors were collected and stored at -70°C until used for isolation of genomic DNA.

The second cohort was provided by the New Mexico Tumor Registry (NMTR) and consisted of 38 archival, paraffin-embedded ductal or lobular carcinomas and matched, histologically normal breast tissues from women who had undergone radical mastectomies or lumpectomies between 1982 and 1993. The histologically normal breast tissues originated from different blocks than the tumor tissues and were obtained at the time of dissection from sites outside the visible tumor margins. Generally, the sections were selected to contain high epithelial cell fractions.

The third cohort was obtained from the University of New Mexico Solid Tumor Facility and consisted of 48 frozen archival invasive ductal or lobular carcinomas from women who had radical mastectomies or lumpectomies between 1982 and 1993. Unlike cohorts 1 and 2, matched, histologically normal breast tissues were not available for the tumors in cohort 3.

The fourth cohort was obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN) and contained 20 normal, disease-free breast tissue samples from women undergoing reduction mammoplasty (NBRST-RM). In addition, peripheral blood lymphocytes (PBLs) were obtained from 59 women previously diagnosed with breast cancer. The women ranged in age from 25 to 74 years, with a mean of 53 years. All tissues used in this study were anonymous, and experiments were performed in accordance with all federal guidelines as approved by the University of New Mexico Health Science Center Human Research Review Committee.

TC assay

Telomere length measurements can be affected by both extraneous factors, such as tissue specimens' age and means of preservation and storage, and inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To minimize the confounding effects of extraneous factors, we previously described a slot blot method for titrating the TC in fresh, frozen or paraffin-embedded tissues up to 20 years old.^{46,47} TC measured by this method is directly proportional to telomere length measured by Southern blot.⁴⁷ However, in contrast to Southern blotting, the TC assay can be performed with as little as 5 ng of genomic DNA,⁴⁶ and is insensitive to fragmentation of DNA to less than 1 kb in length.⁴⁷ Thus, there is excellent agreement between TC measured in paired tissues stored either frozen, or formalin-fixed in paraffin at room temperature.^{28,30} Therefore, TC is a sensitive and convenient proxy for telomere length, particularly for applications where genomic DNA is fragmented or scant, such as in sections of archival, paraffin-

embedded tissues comprising the second cohort of breast tumors, which contains specimens that are over 20 years old.

TC was measured as described previously.⁴⁶ Briefly, DNA was isolated from frozen or paraffin-embedded tissues and blood samples, using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56°C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5'-TTAGGG-3')₄-FAM (IDT, Coralville, IA), was hybridized to the genomic DNA, and the membranes were washed to remove nonhybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP[®]-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm[®] for 2–10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images, using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal of three replicate tumor DNAs compared to the same amount of a placental DNA standard (typically 20 ng). In addition to placental DNA, DNA purified from HeLa cells, which has approximately 30% of placental TC was frequently included to confirm the reproducibility of the assay.

AI assay

DNA (approximately 1 ng) was amplified using the AmpFISTR Identifier PCR Amplification Kit (Applied Biosystems, Foster City, CA), using the manufacturer's protocol. Each multiplex PCR reaction amplifies 16 short tandem repeat (STR) microsatellite loci from independent locations in the genome (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA). Each of the PCR primers is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, allowing the simultaneous resolution of 16 amplicons of similar size. PCR products were resolved by capillary gel electrophoresis and detected using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA). The height of each fluorescence peak in the electropherograms was quantitated using the ABI Prism GeneScan and Genotype Analysis software (Applied Biosystems, Foster City, CA) and a ratio of the peak heights of each pair of heterozygous allelic amplicons was calculated. By convention, the allele with the greater fluorescence intensity was designated the numerator. Thus, the ratio was always ≥ 1.0 , with 1.0 representing the theoretical ratio for normal alleles.

Statistical analysis

Statistical analyses were performed using the JMP[®] statistical package (SAS Institute, Cary, NC), choosing a significance level of 0.01. The nonparametric two-sided Wilcoxon/Kruskal-Wallis log rank test was used to determine the comparative distribution of TC and AI in the breast tumor and TA-HN tissue specimens, as well as associations between TC and AI in the paraffin-embedded breast tumor samples of cohort 2.

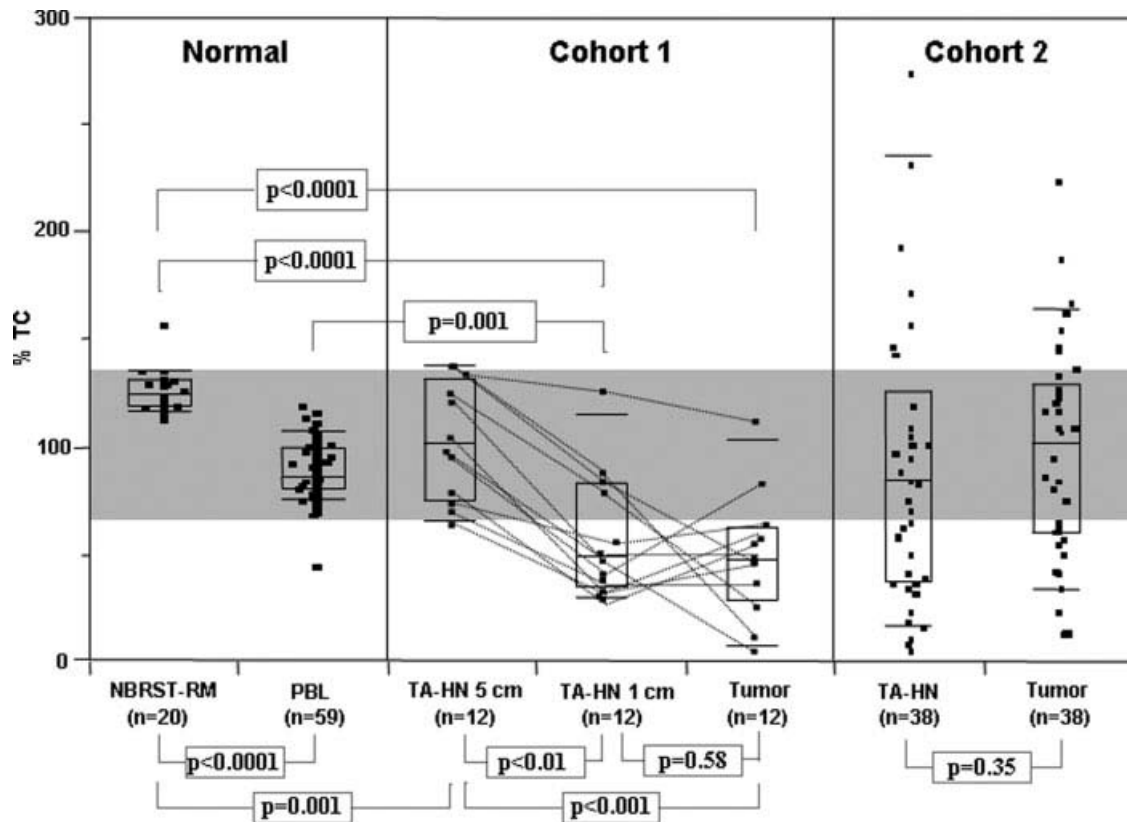


FIGURE 1 – Distribution of telomere DNA content (TC) in disease-free normal breast tissues from reduction mammoplasties (NBRST-RM), in peripheral blood lymphocytes (PBL), and in the breast tumor cohorts 1 and 2, including their tumor-adjacent histologically normal (TA-HN) tissues. TA-HN was excised at 1 and 5 cm from the tumor margin in cohort 1, and at unknown distances from the tumor margin in cohort 2. The number of tissues analyzed is indicated (n). TC is expressed as percentage of TC in placental control. The boxes represent group median (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles, respectively. In cohort 1, TC values of the individual matched samples are connected by thin lines. The gray shaded area indicates 95% of TC measurement for all normal specimens (NBRST-RM and PBLs). The p -values indicate comparisons between different tissue cohorts calculated by the two-sided Wilcoxon Kruskal/Wallis rank sums test. Additional statistical comparisons are mentioned in the text. *Note:* (i) Although the data points are horizontally shifted, some are still overlapping, and therefore not visible; (ii) due to the scale of the figure, two data points at values of 404% and 480% in the TA-HN set of cohort 2 are not shown.

Results

TC in normal breast tissues

To define the normal range of TC in disease-free breast tissues, the TC, a proxy for telomere length,^{46,47} was measured in normal breast tissues obtained from 20 women (mean age 29) undergoing reduction mammoplasty (NBRST-RM). TC ranged from 114% to 158%, with a mean of 127% and a median of 126%, of TC in the placental DNA standard (Fig. 1). The interquartile variation (IQR), a statistical measure of the dispersion of the data, was only 12%, indicating little variation in telomere length in normal breast tissue. For comparison, TC was also measured in PBLs from 59 women (mean age 53) with a previous diagnosis of breast cancer. TC in PBLs ranged from 46% to 120%, with a mean of 90%, a median of 87% and an IQR of 19%, of the standard. The mean TC in normal breast was significantly higher than mean TC in PBLs ($p > 0.0001$). However, greater than 95% of all normal specimens (NBRST-RM and PBLs) had TC values within 70–137% of the standard. This range is interpreted to include the effects of all extraneous and inherent factors on observed TC in normal tissue, including age, tissue site, sample source and experimental variation.

Histology of cancerous and adjacent histologically normal breast tissues

The histologies of the tissues comprising two representative cases from the two independent cohorts of breast tumor tissues and matched tumor adjacent histologically normal (TA-HN) tis-

sues are shown in Figure 2. The first cohort was composed of 12 sets of breast tumor tissues and TA-HN tissues excised 1 cm (TA-HN-1) and 5 cm (TA-HN-5) from the tumor margins. Frozen sections were stained with hematoxylin and eosin and examined microscopically. Sections of the tumors contained variable amounts of infiltrating carcinoma and ductal carcinoma *in situ* (Fig. 2A and 2D). In contrast, both TA-HN-1 and TA-HN-5 tissues had normal architecture, lobular units, ducts, and adipose tissue (Fig. 2B, 2C and 2E, 2F, respectively). Unlike the first cohort, which was composed of snap frozen tissues derived from contemporary mastectomies, the second was composed of paraffin-embedded archival tissues derived from women who had radical mastectomies or lumpectomies between 1982 and 1993. Fig. 2 shows two representative pairs of hematoxylin and eosin stained tumor (Fig. 2G and 2I) and TA-HN tissues (Fig. 2H and 2J). Infiltrating carcinoma can be seen in the tumors, while the TA-HN tissues show normal lobular architecture. Although tumor and TA-HN tissues comprising the second cohort came from different paraffin blocks, and the TA-HN tissues were obtained from sites outside the visible tumor margins, the exact distances between the sites of the TA-HN tissues and the tumors' margins are not known.

TC in tumor and adjacent histologically normal breast tissues

The spatial distribution of TC was examined in the 12 groups of breast tissues comprising the first cohort and compared with TC in the normal, disease-free breast tissues from radical mastectomy (Fig. 1). The mean TC values in the TA-HN-5 and TA-HN-1 tissues

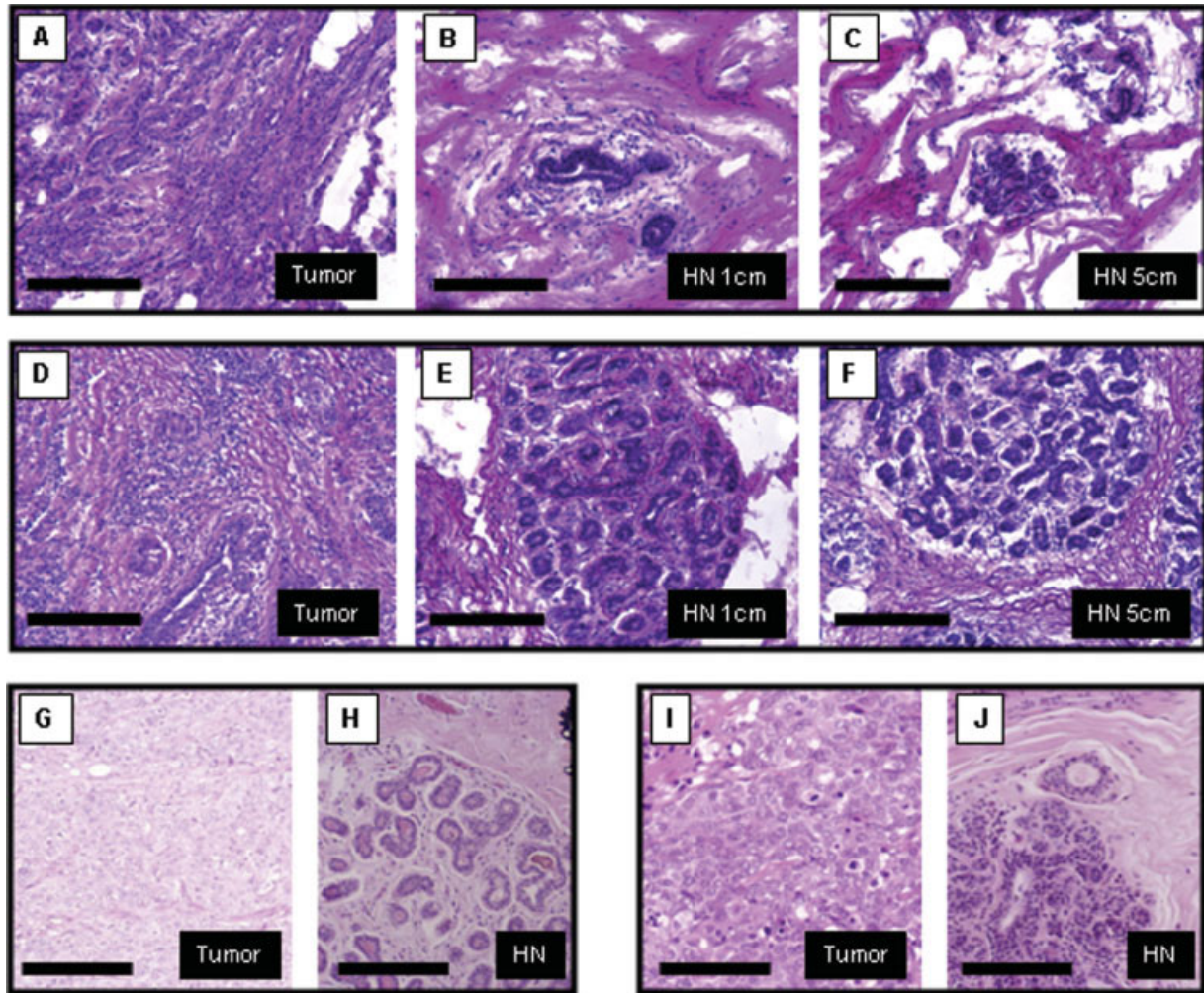


FIGURE 2 – Hematoxylin and eosin staining of human breast tissue sample sections. Two representative cases from the first (A–F) and second (G–J) cohorts are shown. Abnormal architecture with fields of infiltrating ductal carcinoma and ductal carcinoma *in situ* are seen in the tumor sections (A, D, G and I). Normal lobular and ductal architecture and adipose tissue are seen in the tumor-adjacent tissues at the indicated distance from the visible tumor margin (first cohort: B, C and E, F), or at unknown distances (second cohort: H and J). HN, histologically normal tissue; bars represent 200 μ m.

were 101% and 66% of TC in the normal placental DNA standard, respectively. The mean TC value in tumors was 59%. Although the mean TC in TA-HN-5 tissues was significantly less than in NBRST-RM tissues ($p = 0.001$), it was not significantly different than the mean TC in PBLs from women of similar age ($p = 0.16$). Moreover, TC values in each of the TA-HN-5 tissues were within the range that defined >95% of all normal tissues. Since telomere length decreases with age,^{48,49} it is likely that the difference between TC in the normal and TA-HN-5 tissues is due to the different ages of the two groups of women (27 vs. 49 years).

In contrast, mean TC in TA-HN-1 tissues was significantly less than TC in NBRST-RM tissues ($p < 0.0001$) and PBLs ($p = 0.001$), and TA-HN-5 tissues ($p < 0.01$). Mean TC in tumors also was significantly less than those in NBRST-RM tissues ($p < 0.0001$), PBLs ($p < 0.0001$) and TA-HN-5 tissues ($p < 0.001$). However, mean TC in tumor and TA-HN-1 tissues was indistinguishable ($p = 0.58$). Consistent with these findings, TC was, on average, 35% lower in each TA-HN-1 sample than in the paired TA-HN-5 sample, while the differences in TC between the TA-HN-1 and matched tumor specimens were varied, encompassing decrease, stabilization, and increase of TC with an average change of only 3% (lines in middle panel of Fig. 1). In total, TC values in 8 of 12 specimens of TA-HN-1 and 10 of 12 specimens of paired

tumor tissues were outside the range that defined >95% of all normal tissues (NBRST-RM and PBLs).

Similarly, TC distribution was examined in a second, independent cohort (Fig. 1). Although the distributions of TC values in the 38 matched pairs of TA-HN and tumor tissues were broader than those measured in the first cohort (IQR = 88% and 69%, respectively), 16 of 38 TA-HN and 14 of 38 tumor specimens, respectively, had TC values less than those found in NBRST-RM tissues and PBLs, and only 9 of 38 TA-HN and 7 of 38 tumor specimens had TC values exceeding those found in all normal tissues (NBRST-RM and PBLs). A similar TC distribution was observed in a third collection of 48 frozen breast tumors (Table II), and in a collection of archival tumor and matched TA-HN prostate tissues, each collected between 1982 and 1993.²⁸ As observed in the comparison between tumor and TA-HN-1 specimens in the first cohort, there was no difference in mean TC in tumors and TA-HN tissues ($p = 0.35$). However, there was greater heterogeneity in the samples of the second as compared to the first cohort. Nevertheless, data from both cohorts are consistent with the conclusion that significant telomere attrition, comparable to that observed in tumors, occurs in TA-HN breast tissue. Significant telomere attrition (to a level outside the range seen in >95% of all normal tissues) occurred (i) in almost 50% (24/50) of TA-HN-1 and TA-HN

specimens, (ii) at sites at least 1 cm from the tumors' margins, and (iii) since TC is measured in bulk tissue that has not been microdissected, in a substantial fraction of the cells in the samples.

AI in tumor and adjacent histologically normal breast tissues

To investigate the extent of genomic instability in cohorts 1 and 2, tumor and TA-HN tissues were screened for AI at 16 unlinked microsatellite loci. Unlike the TC assay, which utilizes a slot blot methodology to titrate the quantity of telomere DNA in a defined amount of genomic DNA, the AI is defined by the ratio of the peak heights of allelic amplicons after PCR. Thus, it is unlikely that inherent or extrinsic factors that affect measurement of TC would similarly affect the determination of AI. To establish a baseline for the incidence of AI in normal breast tissue, 201 heterozygous loci in the 20 specimens of NBRST-RM tissues were analyzed by this approach. The mean peak height ratio was determined to be 1.18 (SD = 0.166). On the basis of these values, a highly conservative, operational definition of AI was established as a ratio of peak heights ≥ 1.68 , *i.e.*, the mean + 3.0 SD. This threshold excluded more than 99% of the allelic ratios observed in the NBRST-RM tissues, and established a baseline incidence of 0.1 unbalanced loci per specimen of normal breast tissue. As shown in Figure 3, a virtually identical value, 0.08 loci per specimen, was measured in the TA-HN-5 tissues. In contrast, the mean numbers of unbalanced loci in the TA-HN-1 and tumor tissues were 0.42 and 1.25 loci per specimen, respectively, approximately 5 and 15 times higher than the

incidence in the TA-HN-5 tissues. The baseline incidence of 0.1 unbalanced loci per specimen predicts that approximately 10% and 1% of normal tissues will have one and two unbalanced loci, respectively. Consistent with this prediction, 3 of 20 and 1 of 12 NBRST-RM and TA-HN-5 tissues, respectively, had one site of AI. Only one of more than 120 normal samples we have analyzed to date had 2 unbalanced loci, and none had more than 2 unbalanced loci. Accordingly, neither the NBRST-RM nor the TA-HN-5 specimens had more than one unbalanced locus. In contrast, one TA-HN-1, and 5 tumor tissues had 2 or more unbalanced loci. These data are consistent with the conclusion drawn from the TC analysis that both tumors and TA-HN-1 tissues are genetically distinct from TA-HN-5 tissue, and that both are genetically unstable.

This conclusion is further supported by results obtained with the second cohort. Microsatellite alleles were successfully amplified in 23 pairs of the 38 samples. As with the TC determinations, the distribution of the numbers of unbalanced loci was much broader in the second cohort than in the first. The mean numbers of unbalanced loci in the TA-HN tissues and matched tumors were 2.61 and 2.48 loci per specimen, respectively (Fig. 3). The mean numbers of unbalanced loci in TA-HN and tumor tissues were significantly greater than the numbers in either NBRST-RM or TA-HN-5 tissues ($p < 0.01$). The extent of AI in the tumors and their matched TA-HN tissues of the second cohort were indistinguishable ($p = 0.88$). Significantly, 74% (17/23) of TA-HN tissues and 70% (16/23) of matched tumors had 2 or more sites of AI, and 57% (13/23) and 40% (9/23), respectively, had 3 or more sites. Like the TC measurements, the independent measurement of AI, performed in two independent cohorts of paired breast tissues, indicates that at least 1 unbalanced locus is present (i) in more than 74% (26/35) of TA-HN-1 and TA-HN specimens, (ii) at sites at least 1 cm from the tumors' margins and (iii) since AI was measured in bulk tissue that was not microdissected, and the threshold for detecting AI requires that approximately 40% of the cells have lost the specific allele (see later), specific sites of AI are present in a substantial fraction of the cells.

Conservation of unbalanced alleles in tumor and adjacent breast tissues

To investigate the possibility that TA-HN and tumor tissues represented early and late stages, respectively, in the clonal evolution of the cancers, we measured the frequency of conservation of unbalanced loci in the 2 cohorts of paired tumor and TA-HN tissues. As shown in Figure 4, in the first cohort, 2 of the 6 (33%) sites of AI present in TA-HN tissues were conserved in the paired tumors (left panel). Likewise, in the second cohort, 21 of the 60

TABLE II - TC VALUES IN NORMAL, TUMOR AND TUMOR ADJACENT, HISTOLOGICALLY NORMAL (TA-HN) TISSUES¹

	N	Median	Mean	Min	Max	IQR
Normal tissues						
NBRST-RM	20	126	127	114	158	12
PBL	59	87	90	46	120	19
Cohort 1						
TA-HN-5	12	100	101	70	128	44
TA-HN-1	12	59	66	43	119	38
Tumor	12	57	59	24	108	27
Cohort 2						
TA-HN	38	85	106	6	480	88
Tumor	38	102	98	14	224	69
Cohort 3						
Tumor	48	105	118	65	247	60

IQR, interquartile range; NBRST-RM, normal breast tissue from reduction mammoplasty; PBL, peripheral blood lymphocytes.

¹Data from Figure 1.

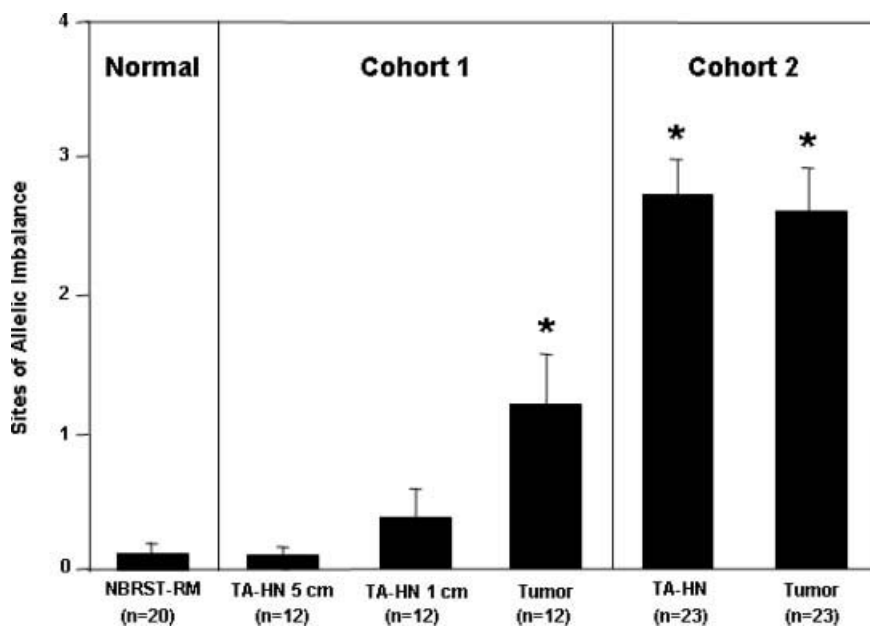


FIGURE 3 - Extent of allelic imbalance (AI) in disease-free normal breast tissues from reduction mammoplasties (NBRST-RM), and in the breast tumor cohorts 1 and 2, including their tumor-adjacent histologically normal (TA-HN) tissues. TA-HN was excised at 1 and 5 cm from tumor margin in cohort 1, and at unknown distances from the tumor margin in cohort 2. The number of tissues analyzed is indicated (*n*). The bars indicate the mean number of unbalanced loci \pm standard errors. The stars indicate statistically significant differences ($p < 0.01$) from both NBRST-RM and TA-HN-5 (two-sided Wilcoxon Kruskal/Wallis rank sums test).

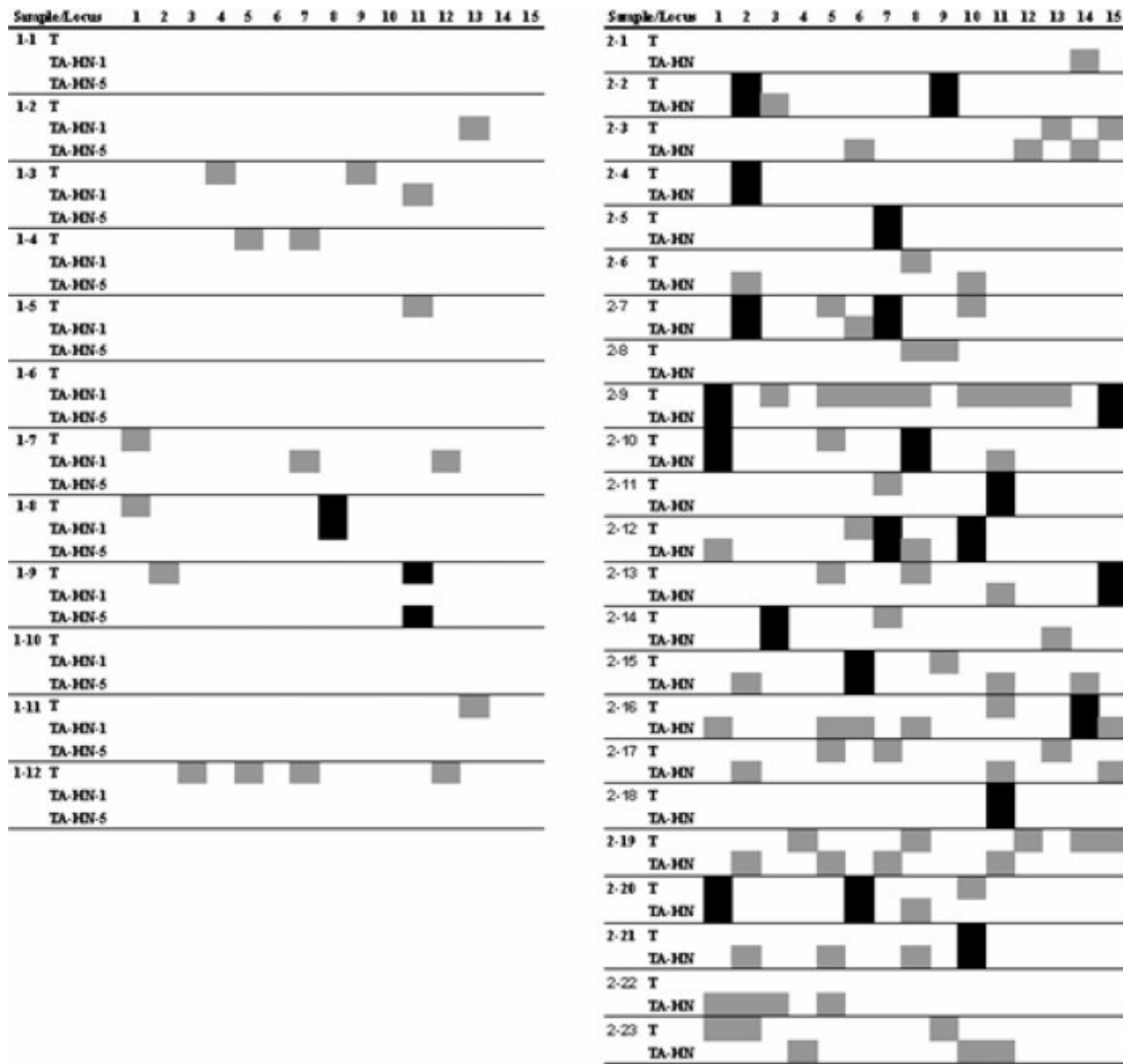


FIGURE 4 – Conservation of unbalanced alleles in matched tumor (T) and tumor-adjacent histologically normal (TA-HN) breast tissues of cohort 1 (left panel) and cohort 2 (right panel). Sites of allelic imbalances are indicated by gray boxes; sites of allelic imbalances conserved between tumor and TA-HN tissues are indicated by black boxes. The unlinked chromosomal loci are designated 1–15 and are as following (1) D8S1179, (2) D21S11, (3) D7S820, (4) CSF1PO, (5) D3S1358, (6) TH01, (7) D13S317, (8) D16S539, (9) D2S1338, (10) D19S433, (11) vWA, (12) TPOX, (13) D18S51, (14) D5S818, (15) FGA. *Note:* Homozygous amelogenin (all female samples) is not shown.

(35%) sites of AI present in TA-HN tissues were conserved in the paired tumors (right panel). The odds of this occurring by chance are estimated to be approximately 3×10^{-2} and 10^{-7} for the first and second cohorts, respectively.

Association between TC and AI in breast tumor tissues

Since telomere attrition is a source of genomic instability, and since we observed telomere attrition and increased AI in breast tumors, we determined the association between TC and AI (Fig. 5). For this analysis, microsatellite alleles were successfully amplified in 30 of the 38 breast tumor samples of cohort 2. Non-parametric 2-sided Wilcoxon/Kruskal–Wallis log rank analysis revealed a significant difference in TC in tumors with high (≥ 3 sites) as compared to low (≤ 2 sites) AI ($p = 0.002$).

Discussion

Although mechanistic insights into the molecular pathology of sporadic breast cancers are increasing, the question of how carcinogenesis is initiated in human breast tissues remains largely unanswered.^{50–53} However, it is widely accepted that genomic instability is a prerequisite of virtually all tumors, including breast

cancers, and that this instability facilitates the accumulation of further genetic alterations that result in cancer progression through clonal expansion of cells with a proliferative advantage.^{1–3,51–53}

Two independent, quantitative measures of genomic instability, TC and AI, were used in this study to demonstrate that genomic instability occurs in histologically normal breast tissues adjacent to the corresponding tumors. These studies show that shortened telomeres (to a level outside the range seen in $>95\%$ of all normal tissues) and unbalanced allelic loci are present (i) in 50–75% of TA-HN and TA-HN-1 specimens, (ii) at sites at least 1 cm from the tumor margins and (iii) in a substantial fraction of the cells comprising the TA-HN tissue. This finding parallels our previous studies on tumors of the prostate and their matched TA-HN tissues,²⁸ and is in agreement with the work of previous investigators who reported that genetic alterations, including telomere attrition and loss of heterozygosity, occur in histologically normal tissues adjacent to breast tumors.^{34–38,41–44} In these previous studies, the sites of telomere attrition, loss of heterozygosity and AI were physically distant from one another and from the tumors, albeit in most cases at undefined distances from the corresponding tumor lesions.^{24,42–44} In contrast, and to our knowledge, the findings in cohort 1 represent the first

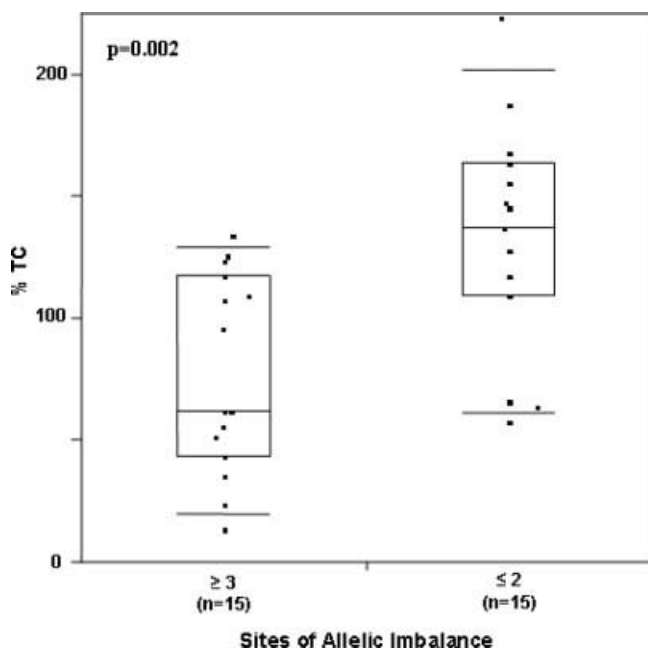


FIGURE 5 – Association between telomere DNA content and allelic imbalance in 30 breast tumor samples of cohort 2. The samples were dichotomized according to the number of genomic sites affected by allelic imbalance, i.e. ≥ 3 or ≤ 2 sites. The number of tissues analyzed is indicated (n). TC is expressed as percentage of TC in placental control. The boxes represent group median (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines below and above boxes indicate 10th and 90th percentiles, respectively. The nonparametric two-sided Wilcoxon/Kruskal–Wallis log rank test was used to assess the statistical significance of the difference between the means.

study in breast cancers that analyzes genomic instability at defined distances (1 and 5 cm) from the visible tumor margins. Consequently, this study reveals that genomic instability in tumor adjacent, histologically normal breast tissues is a function of distance from the tumor lesion, showing decreasing extent of genomic instability with increasing distance from the tumor margin. One explanation for these findings is that breast tumor cells exert a transforming effect on surrounding cells, leading to genetic alterations in adjacent tissues, as has been proposed for prostate cancer cells.^{54,55} However, we prefer the alternate hypothesis, that breast epithelial carcinogenesis occurs at higher frequency in fields of cells with elevated genomic instability. This is supported by our observation that the occurrence of two independent markers of genomic instability, telomere attrition and unbalanced allelic loci, are highest in the tumor lesions and decrease with increasing distance from the tumor. In addition, analysis of tumors reveals an association between TC and extent of AI. Thus, we argue that telomere attrition induces genomic instability in breast tissues, and while this may not necessarily be apparent in histologically normal precancerous tissue, it is strongly displayed in tumor lesions.

Although similar conclusions can be drawn from the TC and AI analyses in each of the two cohorts, the range of TC values and the number of unbalanced loci per specimen were both greater in the second cohort. In this context, it is important to emphasize that both TC and AI reflect the average TC and peak height ratios in the cells comprising the sample; they do not provide information about the variability of TC or AI *between* individual cells. Consequently, the ability to detect specific changes in TC or AI diminishes as the number and types of cells in the sample increases. On the basis of the DNA yields, we estimate that there were approximately 20 times more cells in the samples comprising the first cohort (median $\sim 10^6$ cells), than the second cohort (median $\sim 5 \times 10^4$ cells). This difference reflects the relative amounts of tissue available from the fresh surgical specimens comprising the first cohort versus the sec-

tions of paraffin-embedded tissue blocks comprising the second cohort. This consideration is particularly significant in the case of the AI assay. On the basis of theoretical considerations and mixing experiments (data not shown), we estimate that imbalance at a specific locus must occur in $\sim 40\%$ of the cells in the sample to generate an allelic ratio of 1.68, the threshold for significance used in these studies. Thus, sites of AI that are not prevalent in the cell population are not detected, even if there are many such individual sites. In this context, it is not surprising that specific sites of AI are detectable in breast tumors, which evolve clonally.⁵¹ However, it is remarkable that AI is detected in TA-HN tissue, as it not only reflects underlying genomic instability, but also requires *clonal* expansion of genetically altered, premalignant cell clones within histologically normal breast tissues. This interpretation is further corroborated by the fact that more than a third of unbalanced alleles in adjacent, histologically normal tissues are conserved in the matched tumors. The latter has important practical implications, as it indicates that it is not necessary to micro-dissect tissues, for example using laser capture microscopy, to detect genomic instability, using the assays described in the present study. In fact, these assays allow the selective detection of changes in cell clones undergoing expansion because of proliferative advantages.

Taken together, our results are in agreement with the concept of “field cancerization,” introduced by Slaughter and colleagues in 1953,⁵⁶ and more recently reviewed by others.^{57–59} These authors developed the term to explain the multifocal and seemingly independent areas of histologically precancerous alterations occurring in oral squamous cell carcinomas.⁵⁶ Organ systems in which field cancerization has been implied include lung, colon, cervix, bladder, skin and breast.⁵⁷ The concept of field cancerization has also been used to explain the occurrence of genetic and epigenetic mosaicism in cancer precursor tissues.⁶⁰ Based on our results, we propose to extend the concept of field cancerization to genetic alterations in otherwise histologically normal breast tissues, and our study is the first to include TC.

In head and neck squamous carcinoma, field cancerization has been shown for relatively large tissue areas, i.e. up to 7 cm in diameter.⁶¹ It is thus not surprising that our data show extensive field cancerization in tissues 1 cm outside breast tumor margins. In the present study, TC was also different between disease-free NBRST-RM tissues and TA-HN tissues excised at 5 cm from the tumor margin. However, TC was similar in TA-HN-5 tissues and PBLs from women of similar age. Since telomere length decreases with age,^{48,49} the observed difference in TC between NBRST-RM and TA-HN-5 tissues is likely due to the age discrepancy between the two cohorts of women (27 vs. 49 years).

The existence of fields of genomic instability that support tumorigenic events also has important clinical implications. First, such fields could give rise to clonal selection of precursor cells that ultimately lead to the development of cancer.⁶² In this context, our recent studies have identified the presence of telomerase-positive cell populations within histologically normal tissues adjacent to breast tumors that could represent fields of premalignant cells.⁴⁵ Second, the presence of such fields, even after surgical resection of primary tumors, may represent an ongoing risk factor for cancer recurrence or formation of secondary lesions, which occurs in up to 22% of women undergoing breast conservation therapies for small invasive and noninvasive breast cancers.^{58,63,64} For these reasons, our study has practical implications for the assessment of appropriate tumor margins for breast cancer surgical procedures, secondary treatment options and prognosis, possibly including the risk for the development of new primary tumors in the contra-lateral breast.^{65–67} Thus, our study also suggests that evaluation of surgical margins should include molecular, in addition to histological, techniques, thus warranting further investigations.

Acknowledgements

We thank Myra Zucker from the University of New Mexico Pathology Laboratory for excision of the fresh breast tissues. We thank Kelly Salceies from the Human Tissue Repository for preparing and staining breast tissue sections.

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Preclinical study

Telomere content correlates with stage and prognosis in breast cancer

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Key words: breast cancer, genomic instability, metastasis, prognosis, telomere, TNM staging

Summary

Purpose. To evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissue correlates with TNM staging and prognosis.

Experimental design. Slot blot assay was used to quantitate TC in 70 disease-free normal tissues from multiple organ sites, and two independent sets of breast tumors containing a total of 140 samples. Non-parametric Rank-Sums tests, logistic regression and Cox proportional hazards models were used to evaluate the relationships between TC and tumor size, nodal involvement, TNM stage, 5-year survival and disease-free interval.

Results. TC in 95% of normal tissues was 75–143% of that in the placental DNA standard, whereas only 50% of tumors had TC values in this range. TC was associated with tumor size ($p=0.02$), nodal involvement ($p<0.0001$), TNM stage ($p=0.004$), 5-year overall survival ($p=0.0001$) and 5-year disease-free survival ($p=0.0004$). A multivariable Cox model was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group (>123% of standard), low TC (<101% of standard) conferred an adjusted relative hazard of 4.43 (95% CI 1.4–13.6, $p=0.009$). Receiver operating characteristic curves using thresholds defined by the TC distribution in normal tissues predicted 5-year breast cancer-free survival with 50% sensitivity and 95% specificity, and predicted death due to breast cancer with 75% sensitivity and 70% specificity.

Conclusions. TC in breast cancer tissue is an independent predictor of clinical outcome and survival interval, and may discriminate by stage.

Introduction

It is estimated that in the US in 2005 more than 200,000 women were diagnosed with breast cancer, and approximately 40,000 women died from this disease. Micrometastasis (metastatic cells that have escaped the primary tumor, but are currently undetectable) are a primary cause of breast cancer recurrence and mortality. Although TNM (Tumor size-Nodal involvement-Metastasis) is among the most informative of current prognostic markers for breast cancer [1–2], it often fails to discriminate between women who will have favorable and poor outcomes [1–5]. Thus, it is important to develop new markers that accurately predict the

likelihood of breast cancer recurrence at the time of diagnosis.

Nearly a century ago, Boveri proposed that cancer resulted from altered genetic material. It is now widely accepted that genomic instability – the amplification, loss or structural rearrangement of a critical gene or genes – occurs in virtually all cancers [6]. The phenotype of a tumor is a reflection of its gene expression. Therefore, mechanisms that generate genomic instability, and thereby alter gene expression, play direct roles in tumor progression, including the development of aggressive tumor phenotypes like micrometastasis. Telomere dysfunction is one mechanism of generating genomic instability [7–9]. Telomeres are nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination [10–12]. Due to incomplete replication, telomeres are shortened during each round of cellular replication [13]. Telomere shortening

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may also be a consequence of double-strand DNA breaks, or changes in either the expression or function of any of the numerous proteins required for telomere maintenance [14–16]. Critically shortened, dysfunctional telomeres are prone to chromosome fusion and breakage [17], and in normal somatic cells lead to p53-dependent senescence and apoptosis [18]. However, these mechanisms are inactivated in cancer cells, for example, through p53 and Rb mutations. The direct relationship between dysfunctional telomeres, genomic instability and altered gene expression implies that tumors with the shortest telomeres have the most unstable genomes and, consequently the greatest probability of aberrant gene expression. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations, and therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Accordingly, we and others have postulated that the mean telomeric DNA length in a tumor may provide a surrogate for phenotypic variability and therefore have prognostic potential in tumors [19–21].

There have been several investigations of the relationship between telomere length, or its proxies, and outcome in cancer. The most well characterized of these are in hematological cancers where it has been shown that telomere loss is associated with decreased survival in multiple types of leukemia and myeloma [22–24]. However, there have been few investigations of the prognostic potential of telomere length in solid tumors, which account for the majority of cancer incidence. Primarily, this is because the limited quantity and poor quality of DNA that is typically recovered from archival tissues precludes the use of Southern blotting techniques for the determination of telomere length.

To circumvent these problems, we previously described an alternative approach for measuring telomere length in genomic DNA obtained from fresh, frozen and, most importantly, paraffin-embedded tissues up to 20 years old [25,26]. The content of telomere DNA sequences (TC) in a DNA sample is titrated by hybridization with a telomere specific probe, and then normalized to the quantity of total genomic DNA in the same sample, thus controlling for the differences in DNA ploidy that are frequent in solid tumors. Our previous studies have shown that TC measured by this method is directly proportional to mean telomere length determined by Southern blotting [25]. Thus, TC is a proxy for telomere length and not affected by TTAGGG sequences outside the telomere. However, in contrast to Southern blotting, the TC assay can be performed with as little as 5 ng of genomic DNA and fragmented DNA less than 1 KB in length [25,26]. Therefore, the TC assay is particularly well-suited for analysis of retrospective studies of archival specimens from subjects with known outcomes.

Using this method, we previously demonstrated that reduced TC is associated with metastasis to lymph nodes

in breast cancer [19]. More recently, we reported that TC was an independent predictor of time to prostate cancer recurrence (RH = 5.02) [20]. Short telomeres have also been associated with poor outcomes in neuroblastomas [27] while very long telomeres are a positive prognostic indicator in glioblastoma multiforme [28]. Collectively, these data imply that the extent of telomere loss or gain in tumors may have wide potential as a prognostic marker. However, this conclusion must be considered provisional, as prior studies often were based on small numbers of samples, highly selected patient populations and limited follow-up data using multiple clinical endpoints. In addition, the criteria for defining “long” or “short” telomeres are usually relative, and the relationships between telomere lengths in tumors and true disease-free tissue are often undefined.

In the current investigation, we have used the TC assay to define a normal range of telomere DNA content in breast and other tissues from multiple sites in healthy donors, compared this range to the distribution of TC measured in breast tumor tissues, and evaluated the relationships in breast tumor tissues between TC and TNM stage (and its individual components), 5-year breast cancer survival, and breast cancer-free survival interval following surgical excision of breast carcinoma.

Materials and methods

Tissue samples

Four independent sets of human breast tissues were used in this study. The first set (1982–1993) was comprised of 77 archival frozen and paraffin-embedded breast tumor tissues from women with either invasive ductal or lobular carcinomas who had radical mastectomies ($N = 63$), breast sparing surgery ($N = 11$) or unspecified surgeries ($N = 3$) between 1982 and 1993. The second set (1996–1999) was comprised of 63 archival paraffin-embedded breast tissues from a randomly selected subset of women participating in the population-based Health, Eating, Activity and Lifestyle (HEAL) Study [29]. These women were diagnosed with ductal carcinoma *in situ* (DCIS), invasive ductal carcinomas or invasive lobular carcinomas, and had radical mastectomies ($N = 11$) or breast sparing surgery ($N = 52$) between 1996 and 1999. Clinical data on breast tumors (Tables 1, 2) were ascertained by the New Mexico Tumor Registry (NMTR), a member of the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute. TNM stage was assigned using the 2002 revised criteria [30]. This study was approved by the University of New Mexico (UNM) Human Research Review Committee.

The third set was obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN) and contained disease-free breast tissue from women who had reduction mammoplasty (RM). The fourth set included matched tumor and histologically normal breast (HNB) tissues collected at sites 5 cm from

Table 1. Characteristics of tumor tissues

Set ^a	N	Size (mm)	Node involvement				TNM stage						Number of deaths at 5-years of follow up						
			Median	Mean	Range	Q1	Q3	Yes (%)	No (%)	NA (%)	0 (%)	I (%)	IIA (%)	IIB (%)	IIIA (%)	IIIB (%)	IV (%)	NA (%)	Total (%)
1982-1993	77	30	34	8-80	20	49	51 (66)	25 (33)	1 (1)	0 (0)	11 (14)	20 (26)	25 (32)	16 (21)	1 (1)	2 (3)	2 (3)	27 (35)	17 (22)
1996-1999	63	14	16	0-65	7	20	15 (24)	41 (65)	7 (11)	11 (17)	32 (51)	12 (19)	6 (9)	1 (2)	0 (0)	0 (0)	1 (2)	9 (14)	0 (0)
Combined	140	20	26	0-80	12	34	66 (47)	66 (47)	8 (6)	11 (8)	43 (31)	32 (23)	31 (22)	17 (12)	1 (1)	2 (1)	3 (2)	36 (26)	17 (12)

Abbreviations: N, number of specimens; Q1, Q3, first and third quartile (the difference between Q1 and Q3 is the inter-quartile range, or IQR); NA, not available.

^aTissue sets are described in the Materials and methods section.

^bOf the 10 subjects who died from causes other than breast cancer, six died from other cancers, and one each from dementia, hypertension, pulmonary embolism, and unknown causes.

the visible tumor margins from women receiving full mastectomies at UNM Hospital in 2003 and 2004. To determine the extent to which TC differed as a function of age, tissue of origin and disease-status, buccal cells (BUC) were obtained from healthy male and female college student volunteers and peripheral blood lymphocytes (PBL) were obtained from women previously diagnosed with breast cancer.

Histological review

Paraffin-embedded and frozen tissue sections were stained with hematoxylin and eosin and were examined microscopically. Tumor tissues typically contained from 75-100% tumor cells.

Determination of telomere DNA content (TC)

DNA was extracted from slides cut from frozen or paraffin-embedded tissue, and TC was measured as described [20,26]. Briefly, DNA was isolated from frozen or paraffin-embedded tissues, and blood samples using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56 °C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5'-TTAG GG-3')₄-FAM, (IDT, Coralville, IA) was hybridized to the genomic DNA, and the membranes were washed to remove non-hybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm for 2-10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal from three replicate determinations of each tumor DNA relative to the average chemiluminescent signal in the same amount (typically 20 ng) of a reference DNA standard (placental DNA). DNA purified from HeLa cells, which have approximately 30% of the TC in placental DNA, and samples prepared without DNA served as positive and negative controls, respectively.

Statistical methods

We compared the distribution of TC for normal and tumor specimens and, within tumor specimens, by tumor size, nodal involvement, and TNM stage using schematic plots and the non-parametric Rank-Sums (Kruskal-Wallis) test. Logistic regression was used to model the fraction of tumors <2 cm in size, node

Table 2. Ages at tissue collection and telomere DNA contents in normal and tumor tissues

Set ^a	N	Age at tissue collection					Telomere DNA content (% placental DNA control)				
		Median	Mean	Range	Q1	Q3	Median	Mean	Range	Q1	Q3
Normal											
RM	20	30	29	15–48	21	36	126	127	114–158	120	132
HNB	12	53	49	26–61	39	59	101	101	70–128	79	124
PBL	12	NA	NA	NA	NA	NA	87	91	71–117	78	106
Buccal	26	NA	NA	NA	NA	NA	110	114	89–148	100	126
Combined	70	36	36	15–61	25	51	116	112	70–158	98	126
Tumor											
HNB Matched	12	53	49	26–61	39	59	57	59	24–108	42	69
1982–1993	77	48	52	31–88	42	60	108	109	36–247	77	126
1996–1999	63	56	59	32–85	48	72	136	148	31–359	98	177
Combined	152	53	55	26–88	45	65	110	121	24–359	76	146

Additional details are found in the text and the legend to Figure 1. Abbreviations: N: Number of specimens, Q1, Q3: first and third quartile (The difference between Q1 and Q3 is the interquartile range, or IQR). NA: Not available.

^aTissue sets are described in the Materials and methods section.

negative status, and at each TNM stage as a function of TC. The results of the logistic regression models are shown as plots of predicted values against TC. We investigated the association between survival and TC using Kaplan–Meier survival plots for three categories of TC, which were based on tertiles of the TC distribution in normal specimens. Death from any cause and death due to breast cancer were evaluated separately in the survival analyses. Cox proportional hazards models were used to control for the confounding effects of TNM stage and age. SAS version 9.1 and JMP (SAS Institute) were used for all analyses. *P*-values < 0.05 were considered to be significant.

Results

Telomere contents in normal tissues

Telomere content can be affected by several inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To evaluate the potential variability in TC arising from inherent properties of tissues, TC was measured in a diverse sampling of 70 specimens of normal tissue from multiple organ sites (Figure 1). Specimens included breast tissue obtained by reduction mammoplasty (RM); histologically normal breast tissues excised from sites 5 cm from the breast tumor margins (HNB), buccal cells from healthy, young men and women (BUC) and PBL from women with a prior diagnosis of breast cancer (PBL). As summarized in Table 2, median TC in HNB and PBL sets (101 and 87%, respectively) were approximately 30% lower than median TC in the RM and buccal specimens (126 and 110%, respectively). Similarly, the median ages for the donors of the HNB set (53 years) was almost twice the median ages of the donors of the RM samples (30 years). Although the ages of the volunteers contributing the

BUC and PBL samples were not collected, the BUC samples were obtained from college students in their early 20s, while the PBL samples were obtained from a subset of a larger study group with a median age of 58 years. Thus, the results are consistent with the accepted view that telomere length in humans decreases as a function of age [13].

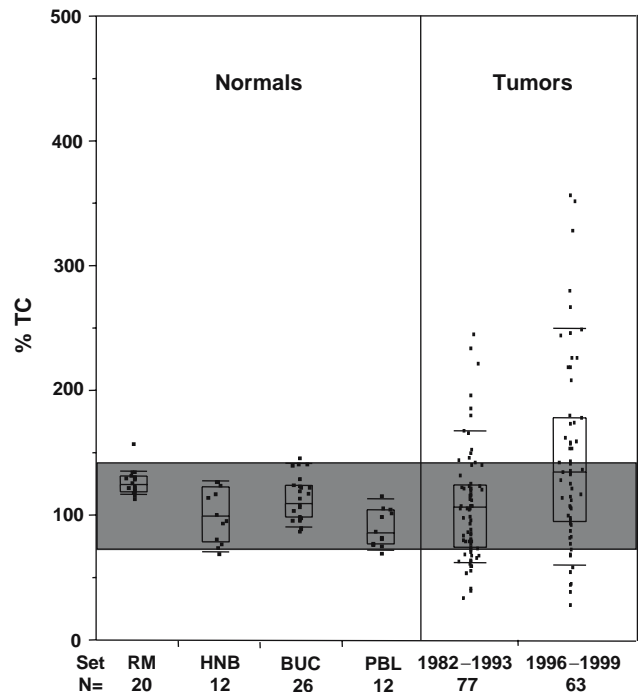


Figure 1. Distributions of telomere DNA contents (TC) in normal and tumor tissues. TC is shown on the y-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the x-axis. The shaded area (75–143% of the placental DNA standard) contains 95% of the TC values in the four sets of normal tissues. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are shown as lines above and below the box.

The inter-quartile range (IQR), a statistical measure of the dispersion of the TC data, was 28% for the combined normal tissues (Table 2). Ninety-five percent of all normal specimens had TC values of 75–143% of the standard (shaded area, Figure 1). In order to assess the extent to which this range was truly representative of normal tissues, we measured TC in a second, independent collection of 60 normal tissues (9 renal, 1 bone marrow, 2 breast, 2 lymph node, 2 prostate, 1 tonsil and 43 PBL). Similarly, 95% of the specimens had TC values within 75–145% of the standard (data not shown). Therefore, the distributions of TC in normal tissues is approximately 75–145%, which includes the effects of all extraneous factors, such as experimental variation, and inherent factors, such as subject's age and health status, the tissue type and source.

Telomere contents in breast tumor tissues differ from normal tissues

Matched tumor tissue was available for the 12 specimens of HNB tissues described above. Although TC in 11/12 of the HNB tissues fell within the expected range for normal tissues, only 2/12 matched tumors had TC within this range (Table 3). On average, TC in tumors was 61% of TC in the matched HNB tissues. TC was measured next in the 140 tumors comprising the 1982–1993 and 1996–1999 tumor sets (Figure 1). The IQR for TC in the two sets of tumor tissues, 49 and 79%, respectively, were substantially greater than the 28% IQR of the normal tissues (Table 2). Fifty-six percent of breast cancer specimens in the 1982–1993 set had TC values within the range that contained 95% of normal tissues, while 23 and 21% had TC values less and greater than the normal range, respectively. Similarly, only 43%

of breast cancer specimens in the 1996–1999 set had TC values within the range that contained 95% of normal tissues, while 14% were below the range and 43% were above. Thus, TC in breast cancer tissues is significantly more heterogeneous than that in normal tissues, reflecting frequent abnormally short and long telomeres.

Telomere contents in breast tumor tissues are associated with TNM stage

As shown in Table 2, mean and median TC differed between 1982–1993 and 1996–1999 tumor sets. A non-parametric Rank-Sums test of this difference in the means (109 and 148%, respectively) was highly significant ($p=0.0008$). There were also highly significant differences between the two sets in the women's ages at diagnosis ($p=0.001$), and their tumor's sizes ($p<0.0001$), nodal involvements ($p=0.0009$) and TNM stages ($p<0.0001$). In order to more directly address a possible relationship between TC and the age at diagnosis, tumor size, nodal involvement and TNM stage, the two tumor sets were combined and these relationships were evaluated by non-parametric Rank-Sums tests (Figure 2a–c) and logistic regressions (Figure 2f–h). In each instance, there were highly significant associations with TC. Approximately 85% of the tumors in the 1982–1993 set were TNM stage IIA or higher; while approximately 66% of tumors in the 1996–1999 set were TNM stage 0 or I (Table 1). This, coupled with the strong association between TC and node status, suggests that TC discriminates across TNM stages. In contrast, there was no detectable association between TC and tumor histology (i.e. ductal versus lobular carcinomas).

Telomere contents in breast tumor tissues are associated with breast cancer survival

We hypothesized that telomere DNA length in a tumor is a surrogate for phenotypic variability and, therefore, atypically long and short telomeres, measured by high and low TC, respectively, are more likely associated with favorable and poor clinical outcomes, respectively. At least 5 years of follow-up data were available for 137 of the 140 women in the 1982–1993 and 1996–1999 sets. The relationships between TC and both overall 5-year survival and breast cancer-free 5-year survival were evaluated by non-parametric Rank-Sums tests (Figure 2d,e) and logistic regressions (Figure 2i,j). Both methods demonstrated highly significant associations between TC and overall 5-year survival ($p=0.0001$, $p<0.0001$, respectively) and breast cancer-free 5-year survival ($p=0.0004$, $p=0.0002$, respectively). The same conclusion was reached when the two tumor sets were analyzed separately (data not shown). In these analyses, the Kruskal-Wallis tests demonstrated that TC in the 1982–1993 group was associated with both overall 5-year survival ($p=0.01$) and breast cancer-free 5-year survival ($p=0.005$). TC in the 1996–1999 set was also associated with overall 5-year survival ($p=0.02$)

Table 3. TC in paired HNB and tumor tissue

Subject ^a	Telomere DNA content (% placental DNA control)		
	HNB (%)	Tumor (%)	T/N (%)
A	95	58	61
B	75	49	65
C	78	70	90
D	102	56	55
E	115	24	21
F	70	65	93
G	128	56	44
H	97	85	88
I	82	63	77
J	118	40	34
K	128	29	23
L	125	108	86
Average	101	59	61

Additional details are found in the text and the legend to Figure 1. T/N is the percent TC in the tumor (T) relative to TC in the paired, histologically normal (HNB) tissues.

^aTissue sets are described in the Materials and methods section.

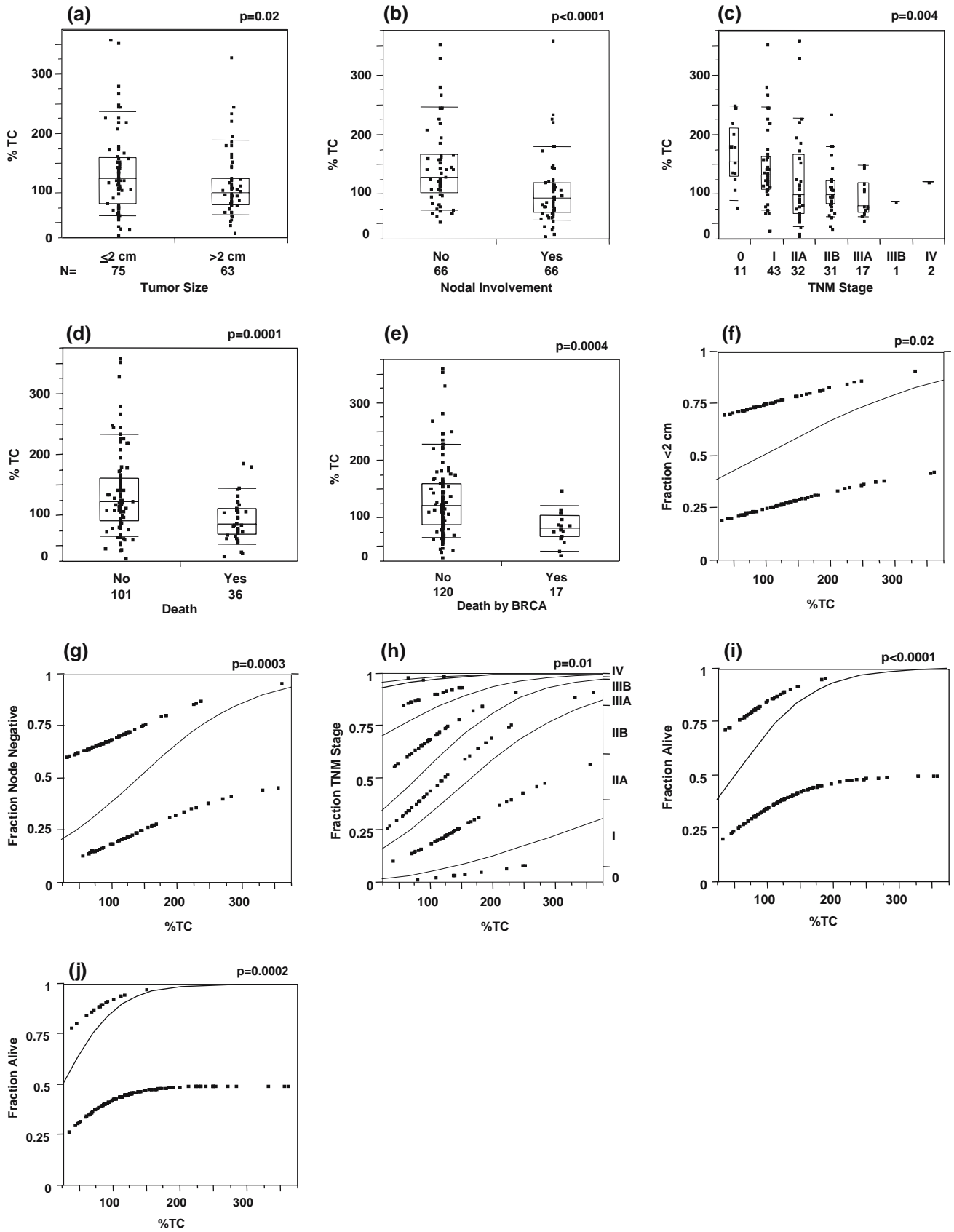


Figure 2. Associations between breast tumors' telomere DNA contents (TC) and tumor size, nodal status, TNM stage and 5 year breast cancer-free survival. Tumor sets 1982–1993 and 1996–1999 were combined and stratified by tumor size (a), nodal status (b), TNM stage (c), overall 5-year survival (d) and breast cancer-free 5-year survival (e). TC is shown on the y-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the x-axis. Statistical significance (*p*) was determined using the 2-sided non-parametric Rank-Sums test. The relationships between TC and tumor size (f), nodal status (g), TNM stage (h) overall 5-year survival (i) and breast cancer-free 5-year survival (j) were also evaluated by logistic regression. Logistic regression estimates the probability of choosing one of the specified parameters (e.g. large vs. small tumors) as a continuous function of TC. In a logistic probability plot, the y-axis represents probability. TC is shown on the x-axis, and is expressed as a percentage of TC in the placental DNA standard. The proportion of small tumors (i.e. <2.0 cm), node negative tumors, TNM stage 0–IV tumors, and survivors are shown on the y-axis. See the legend to Figure 1 for additional details.

however, no members of the 1996–1999 set died from breast cancer within 5 years of surgery (Table 1). Highly significant relationships between TC and overall 5-year survival ($p=0.01$) and breast cancer-free 5-year survival ($p=0.002$) in the 1982–1993 group, and overall 5-year survival in the 1996–1999 set ($p=0.02$) were also detected by logistic regression. Collectively, the data support the conclusion that longer telomeres are protective while shorter telomeres presage poor survival.

The sensitivity and specificity of TC as a predictor of breast cancer-related death was evaluated by analysis of the TC's receiver operating characteristics (Figure 3). TC ranges for the lower, middle and upper tertiles in normal tissues were <101, 101–123, and >123% of standard, respectively. Consistent with the data in Figure 1 demonstrating that many tumors have TC values that are greater or lesser than those typically observed in normal tissues, only 20 and 14% of tumors in the 1982–1993 and 1996–1999 sets, respectively, had TC values

within the range defined by the middle tertile. The 124% cutoff predicted 5 year survival with approximately 50% sensitivity and 95% specificity, while the 100% TC cutoff predicted death due to breast cancer with approximately 75% sensitivity and 70% specificity.

Telomere contents in breast tumor tissues predict breast cancer-free survival interval

The extensive follow up data associated with the 77 tumors in 1982–1993 set (up to 23 years) made it possible to evaluate the effect of TC on breast cancer-free survival. The tumors were grouped using the TC thresholds described above: low TC was defined as less than or equal to 100%, intermediate TC was defined as 101–123%, and high TC was defined as greater than 123%. A Kaplan–Meier plot and Log–Rank test (Figure 4) demonstrated significant differences in the groups' survival intervals ($p=0.013$). This effect is independent of age at diagnosis, nodal involvement and TNM stage (Table 4).

As shown in Table 5, low TC conferred an unadjusted relative hazard of 4.39 (95% CI=1.47–13.08;

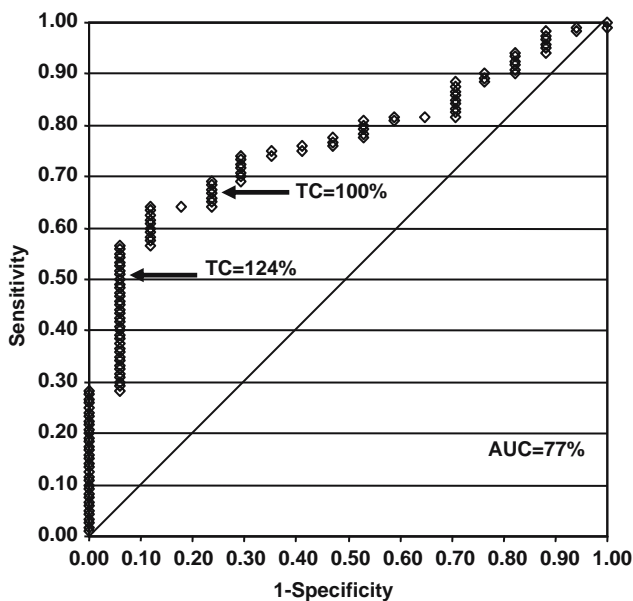


Figure 3. Receiver operating characteristics (ROC) analysis of the relationship between TC in breast tumors and 5 year survival. The specificity and sensitivity of TC as a predictor of 5 year survival following diagnosis of breast cancer was calculated for each value of TC in the combined 1982–1993 and 1996–1999 sets. Seventeen subjects died from breast cancer, and 119 survived for at least 5 years after diagnosis. The plot shows 1-specificity (the false positive rate) on the x-axis and sensitivity (1 – the false negative rate) on the y-axis. Arrows correspond to the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. See text for additional details. The area under the curve (AUC) is 77%.

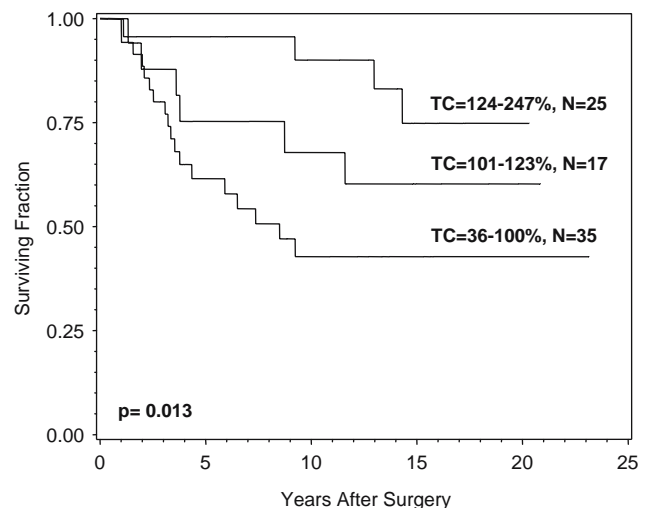


Figure 4. Breast cancer death by telomere DNA content in breast tumors. The 1982–1993 set was divided into three groups based on the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. Breast cancer-free survival interval, in years, is shown on the x-axis and the recurrence-free fraction is shown on the y-axis. The Log–Rank test was used to test the significance of the differences in the groups survival intervals ($p=0.013$). *N* represents the number of subjects in each group. See Materials and methods section for additional details.

Table 4. TNM stage, lymph node involvement, mean age at diagnosis and tumor size by TC level

	TC level					
	36–100%		101–123%		124–247%	
	<i>N</i>	%	<i>N</i>	%	<i>N</i>	%
TNM stage						
I	3	8.6	3	17.6	5	20.0
IIA	11	31.4	2	11.8	7	28.0
IIB	12	34.3	6	35.3	7	28.0
IIIA, IIIB, IV	9	25.7	5	29.4	5	20.0
Unknown	0	0.0	1	5.9	1	4.0
Lymph nodes						
Negative	8	22.9	5	29.4	12	48.0
Positive	27	77.1	12	70.6	12	48.0
Unknown	0	0.0	0	0.0	1	4.0
	<i>N</i>	Mean	<i>N</i>	Mean	<i>N</i>	Mean
Age at diagnosis	35	56.3	17	46.9	25	48.5
Tumor Size (mm) ^a	35	36.1	15	32.1	25	32.1

Abbreviations: TC, telomere DNA content; *N*, number of specimens.

^aSize is measured in longest dimension.

Table 5. Relative hazards and 95% confidence intervals from proportional hazards model of survival from date of diagnosis of breast cancer

	Unadjusted		Adjusted for age, TNM stage	
	RH (95% CI)	<i>p</i> -Value	RH (95% CI)	<i>p</i> -Value
TC level				
36–100	4.39 (1.47, 13.08)	0.0079	4.43 (1.44, 13.64)	0.0094
101–123	2.33 (0.66, 8.27)	0.1900	1.95 (1.54, 7.06)	0.3066
124–247	1.00		1.00	

A proportional hazards model of survival from date of diagnosis of breast cancer and up to 23 years of follow up was used to derive the unadjusted and adjusted relative hazards (RH) associated with each TC group. The adjusted RH was developed using age at diagnosis, TNM Stage and TC as independent predictors of survival. The 95% confidence intervals for RH are shown in parenthesis. Abbreviations: TC, telomere DNA content; RH, relative hazard; CI, confidence interval. See Materials and methods section for additional details.

$p=0.008$) relative to high TC. A multivariable Cox model for the 1982–1993 breast tumor tissue set was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group, low TC conferred an *adjusted* relative hazard of 4.43 (95% CI=1.44–13.64; $p=0.009$). In total these data demonstrate that TC predicts clinical outcome in invasive breast cancer.

Discussion

Telomere DNA content (TC) is a convenient proxy for telomere length that is particularly well-suited for the analysis of samples where DNA is degraded or scant, such as sections from archival, paraffin-embedded tissues. We measured TC values in three independent sets of cancerous breast tissues, compared these to TC in four sets of normal breast, buccal and blood cells, and evaluated the associations of TC with tumor markers and clinical endpoints, including disease-free and overall survival, in two independent cohorts comprising a total of 140 women with invasive breast cancer.

Four principal findings were made from this study. The first is that the range of telomere lengths in each of the three sets of breast tumors, measured as TC, is significantly greater than the range of TC in tissues from disease-free breast, buccal cells and blood cells. Only 17% of all tumors had TC values that were within the range defining the middle tertile of normal tissues, and approximately half of all tumors had TC values greater or lesser than those in 95% of normal tissues. These differences exceed those attributable to the several inherent and extraneous factors that can potentially confound measurements of telomere length, including age, and demonstrate the disparity between the regulation of telomere length in normal and tumor cells. It is significant that TC was associated with age in normal tissues, but not in tumors. This suggests that the extent of telomere attrition and the activities of the compensatory mechanisms that lengthen and stabilize telomeres, such as telomerase-dependant or -independent (“ALT”) processes, occurring in tumor cells are sufficiently large to obscure the underlying, age-dependent differences in telomere length.

Second, TC had significant associations with TNM stage (0 or I versus IIA and higher) and also two of its components: tumor size and nodal status. In contrast to previous studies, and our investigation of prostate tumors, where TC cutoffs were defined arbitrarily [20], TC cutoffs in the present study were derived from the distribution of TC values in normal tissues. Given the small amounts of DNA necessary to measure TC (as little as 5 ng), these results suggest that TC obtained by needle biopsy or fine needle aspirates (FNA) may be used to provide physicians preliminary TNM staging (or nodal involvement) information prior to surgery.

We next demonstrated an association between TC in breast tumor DNA and vital status following surgery. Even though the two tumor sets were not controlled for adjuvant therapies, the relationships between TC and overall 5-year survival and breast cancer-free 5-year survival were highly significant ($p=0.0001$ and $p=c0.0004$, respectively). TC thresholds based on the tertile distributions in normal tissues (described above) predicted 5 year breast cancer-free survival with approximately 50% sensitivity and 95% specificity and death resulting from breast cancer within 5 years of surgery with approximately 75% sensitivity and 70% specificity. Kaplan–Meier plots confirmed that TC was associated with the breast cancer-free interval.

Finally, TC provides prognostic information that is independent of its ability to discriminate disease stage. The relative hazard for death by breast cancer following diagnosis that is conferred by TC values $\leq 100\%$, after controlling for age at diagnosis and TNM stage involvement (RH=4.43), was highly significant ($p=0.009$). This result is nearly identical to our prior finding that the relative hazard for recurrence of prostate cancer following prostatectomy conferred by TC values $\leq 75\%$, after controlling for age at diagnosis, Gleason sum, and pelvic node involvement (RH = 5.02) was also significant ($p=0.013$) [20]. Together, these data support the hypothesis that TC provides independent prognostic information in multiple solid tumor types. We hypothesize that telomere content predicts the likelihood of micrometastasis and, in combination with extant prognostic markers, might have better predictive value than the extant markers alone, thus providing patients and their physicians new information to guide therapeutic decisions.

It is important to point out that all of the analyses reported herein were performed with DNA purified from tumor tissues that had *not* been microdissected. Although histological review of tissue sections indicated that tumor cells typically comprised 75–100% of the samples, the potentially confounding effects of contaminating normal cells in the tumor warrants consideration. In this context, we recently demonstrated that telomere attrition comparable to that in matched prostate and breast tumor tissues occurs in histologically normal tissues at distances at least one centimeter from the visible tumor margins [20,31]. In the latter study, it was estimated that at least 40% of the cells in the tumor

adjacent histologically normal (TAHN) breast tissues were genetically aberrant, and more than a third of unbalanced alleles in the tumor were conserved in matched TAHN breast tissues, implying that the tumor and TAHN cells were derived from the same progenitor. Taken together, these data support the conclusion that TC in tumors and “contaminating” normal cells are comparable, thus precluding the requirement for tissue microdissection.

In summary, we report consistent differences in TC between normal, disease-free and cancerous breast tissues that are statistically significant by tumor characteristics and clinical outcome. We conclude that TC is a marker associated with disease stage and, importantly, appears to be an independent predictor of clinical outcome and survival.

Acknowledgments

This work was supported by research grants DAMD17-01-1-0572 and W81XWH-05-1-0226 to JKG from the DOD BCRP. CAF and CMH were supported by predoctoral training awards, DAMD 17-00-1-0370 and W81XWH-05-1-0273 from the DOD BCRP. JLW and CMH also were supported by an NIH MBRS Award, R25 GM60201, an NIH MARC Award, T34 GM08751, and DOD BCRP Undergraduate Breast Cancer Summer Research Training Program Award, DAMD17-02-1-0513-01. RNB and KBB and data from the HEAL Study were supported by SEER/NCI N01-CN-65034-29. We are indebted to Dr Melanie Royce for critically reviewing the manuscript and her several helpful suggestions.

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Telomere DNA Content Predicts Breast Cancer – Free Survival Interval

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Abstract **Background:** Telomeres are nucleoprotein complexes that protect chromosome ends from degradation and recombination. Critically shortened telomeres generate genomic instability. It has been postulated that the extent of telomere DNA loss is related to the degree of genomic instability within a tumor and therefore may presage clinical outcome. The objective of this investigation was to evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissues predicts breast cancer – free survival interval.

Materials and Methods: Slot blot titration assay was used to quantitate TC in 530 archival breast tumor tissues in a population-based cohort. The relationships between TC, 12 risk factors for breast cancer adverse events (i.e., death due to breast cancer, breast cancer recurrence, or development of a new primary breast tumor), and breast cancer – free survival interval were evaluated by Fisher's exact test, log-rank analysis, and univariate and multivariate Cox proportional hazards models.

Results: TC was independent of each of the 12 risk factors. Ethnicity, tumor-node-metastasis stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy sequence, adjuvant therapy, and TC each conferred significant relative hazards. The best overall multivariate Cox proportional hazards model included TC, p53 status, tumor-node-metastasis stage, and estrogen receptor status as independent predictors of breast cancer – free survival interval ($P < 0.00005$). Low TC ($\leq 200\%$ of standard), relative to the high-TC group ($> 200\%$ of standard), conferred an adjusted relative hazard of 2.88 (95% confidence interval, 1.16-7.15; $P = 0.022$) for breast cancer – related adverse events.

Conclusions: TC in breast cancer tissue is an independent predictor in this group of breast cancer – free survival interval.

Therapeutic management of breast cancer is complicated by the reality that conventional prognostic markers, such as patient age, tumor-node-metastasis (TNM) stage, and hormone receptor status, often do not identify women who will have a local or distant recurrence (1–3). Hence, many women are unintentionally overtreated or undertreated. For example,

approximately one-third of women with breast cancer are node-negative at the time of diagnosis, of whom ~80% and 70% will survive for 5 and 10 years, respectively, if treated with surgery and radiotherapy alone (1). Adjuvant polychemotherapy in node-negative patients with ages <50 years improves 10-year survival from 71% to 78%, whereas in patients with ages 50 to 70 years, adjuvant therapy improves 10-year survival from 67% to only 69% (1). However, because currently available staging and prognostic markers cannot reliably identify the minority of women who will benefit from adjuvant therapy, the NIH/National Cancer Institute and St. Gallen guidelines each recommend adjuvant polychemotherapy for all women with moderate-risk to high-risk breast cancer (2, 3). Consequently, the majority of women with localized tumors have therapy-related side effects and reduced quality of life while gaining no therapeutic benefit (4). Thus, there is a pressing need for new markers that accurately predict the likelihood of breast cancer recurrence.

Tumorigenesis in humans is a multistep process in which successive genetic alterations, each conferring a selective advantage, drives the progressive transformation of normal cells into highly malignant cancer cells (5). Due to incomplete replication, telomeres, the nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination, are shortened during each round of cellular

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Received 2/20/07; revised 7/18/07; accepted 8/23/07.

Grant support: Department of Defense Breast Cancer Research Program grants DAMD17-01-1-0572, W81XWH-05-1-0226, W81XWH-05-1-0273, National Cancer Institute/Surveillance, Epidemiology, and End Results grant NO-1-CN-65034-29 and NCI-PC-05016-20, and NIH grant RR0164880.

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doi:10.1158/1078-0432.CCR-07-0432

replication (6), resulting in a reduction in telomere length with each cycle of chromosome replication (7, 8). Consequently, there is a limit to the number of doublings somatic cells can undergo before telomeres are critically shortened, become dysfunctional, and trigger successive rounds of chromosome breakage-bridge-fusion cycles, thus driving chromosome amplification, loss or structural rearrangement, and, consequently, tumorigenesis (5, 9–12).

The relationship between dysfunctional telomeres, genomic instability, and altered gene expression implies that tumors with the shortest telomeres have the most unstable genomes and, consequently, the greatest probability of aberrant gene expression. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations and, therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Accordingly, several recent studies suggest telomere length may provide independent prognostic information for several solid tumors, including breast cancers (reviewed in ref. 13). However, measurement of telomere length in formalin-fixed, paraffin-embedded (FFPE) tissues that are typically available for retrospective studies is problematic due to the limited quantity and poor quality of the DNA that is recovered. Methods that are not affected by these limitations, such as telomere fluorescence *in situ* hybridization, are not well suited for the high-throughput analyses needed for large sample sets (14).

To circumvent these problems, we previously described a method for measuring telomere length in genomic DNA obtained from fresh, frozen, and, most importantly, FFPE tissues (15, 16). The content of telomere DNA sequences (TC) in a DNA sample is titrated by hybridization on a slot blot with a telomere-specific probe and then normalized to the quantity of total genomic DNA in the same sample, thus controlling for the differences in DNA ploidy that are frequent in solid tumors. TC is particularly well-suited for use with DNA from archival tissues: TC is directly proportional to telomere length measured by Southern blot ($r = 0.904$), can be measured with as little as 5 ng of genomic DNA, is insensitive to fragmentation of the DNA to <1 kb in length, and can be measured successfully in DNA from FFPE tissues stored for up to 20 years at room temperature (15–18).

Using this method, we have recently shown that TC is associated with breast cancer-free survival interval [relative hazard, 4.43; 95% confidence interval (95% CI), 1.44–13.64; $P = 0.009$], controlling for age at diagnosis and TNM stage (17). This study and other investigations (reviewed in ref. 13) provide strong evidence that TC predicts clinical outcome. However, our previous study had a retrospective design (which is more open to bias than the current prospective study), included a limited number ($n = 77$) of specimens collected in the mid 1980s and early 1990s, and was not controlled for the effects of adjuvant treatments and other clinical and prognostic variables. Therefore, it is unknown how TC would perform as a prognostic marker in a contemporary, population-based cohort, in which most tumors are detected by screening at earlier stages and many women elect breast-sparing surgery with adjuvant radiation, chemotherapy, or hormonal therapy.

In the current investigation, we addressed these questions by assessing the relationship between TC and breast cancer-free survival interval in FFPE tumor specimens obtained from 530 members of the New Mexico subset of the National Cancer

Institute/Surveillance, Epidemiology, and End Results Health, Eating, Activity and Lifestyle (HEAL) prospective, population-based cohort (19).

Materials and Methods

Tissue samples. The HEAL study is an ongoing population-based, multicenter prospective cohort study of women diagnosed with breast cancer designed to evaluate the association between body composition, hormones, diet, physical activity, and prognosis over time for non-Hispanic White, Hispanic, and African-American women ascertained through the Surveillance, Epidemiology, and End Results registries³ (19). In New Mexico, incident cases were ascertained by the New Mexico Tumor Registry. Eligibility was based on a first primary breast cancer diagnosis with *in situ* or stages I to IIIA breast cancer (based on the revised 2002 American Joint Committee on Cancer stage groupings; ref. 20) between July 1, 1996 and March 31, 1999, with ages 18 years or more, and residence in one of five centrally located New Mexico counties (Bernalillo, Santa Fe, Sandoval, Valencia, Taos). Women completed a postdiagnosis interview, blood draw, and anthropometric measurements. A total of 998 eligible first primary breast cancer cases were ascertained. Of the eligible cases, 615 patients (61%) chose to participate in the study. Participation rates were 55% for Hispanics and 64% for non-Hispanic Whites. Reasons for nonparticipation or exclusion included physician refusal (3%), unable to locate or interview subject (12%), and subject refusal (24%). Of the 615 total eligible patients for the study, 530 cases (86%) had slides retrieved for subsequent TC analysis, and there was no statistically significant difference in the block retrieval rates between cases with invasive and *in situ* disease. Lymph node status, tumor size, age, chemotherapy, adjuvant therapy, hormonal therapy, and menopausal status were based on medical record abstraction. Lymph node status was based on whether nodes were examined, and the number was identified as positive or negative for cancer. Ethnicity and family history were based on self-report at the time of interview. Coded data, stripped of all personal identifiers (Table 1), were provided by the HEAL investigators (R.N.B. and K.B.B.) and the New Mexico Tumor Registry, as approved by the University of New Mexico Human Research Review Committee. The mean age and follow-up of cohort members were 59.1 (range, 29–89; SD, 12.5) and 6.7 (range, 0.45–9.16; SD, 1.6) years, respectively. At the time of analysis, 83% of the cohort members were alive. Additionally, 85% of the cohort members were free of disease, either at time of analysis or at time of their non-breast cancer-related deaths.

Histologic review. FFPE tissue sections were obtained from the original diagnostic material, stained with H&E and examined microscopically by a surgical breast pathologist. Tissue sections were not microdissected and typically contained from 75% to 100% tumor cells.

Determination of TC. DNA was extracted from four 10- μ m FFPE tissue sections, and TC was measured in known masses, typically 5 to 10 ng, by slot blot titration assay, as previously described (17, 18). TC is expressed as a percentage of the TC in a placental DNA standard measured in parallel. Each measurement was repeated independently thrice and the coefficient of variation for each sample was <10%.

Immunohistochemistry. Immunohistochemistry was done on FFPE breast tumor sections to determine hormone receptor, p53, and HER2/*neu* status. Hormone receptor assays were conducted in laboratories associated with the hospitals, wherein cases were diagnosed. p53 protein expression was evaluated using the anti-p53 monoclonal antibody DO-7 (Santa Cruz Biotechnology), which recognizes both the mutated and wild-type protein (21). p53 tumor suppressor gene mutations occur in 20% to 50% of breast carcinomas (22) and have been reported to be associated with poor prognosis (23). Mutations in p53 are predominantly missense and lead to conformational alterations

³ <http://appliedresearch.cancer.gov/surveys/heal/>

Table 1. Relative hazards of risk factors for breast cancer-related adverse events in the HEAL patient cohort by TC level

Characteristic	All patients				High TC		Low TC	
	<i>n</i>	Percentage (<i>n</i> = 530)	Relative hazard (95% CI)	<i>P</i>	<i>n</i>	Percentage (<i>n</i> = 86)	<i>n</i>	Percentage (<i>n</i> = 444)
Ethnicity								
Non-Hispanic White	408	77	1.0		69	80	338	76
Hispanic	122	23	1.78 (1.11-2.84)	0.017	17	20	106	24
TNM stage								
0 (<i>in situ</i>)	97	18	1.0		17	20	80	18
I	259	49	0.92 (0.46-1.86)	0.820	43	50	216	49
IIA	115	22	1.87 (0.91-3.85)	0.087	19	22	96	22
IIB	41	8	3.73 (1.71-8.13)	0.001	5	6	36	8
IIIA	5	1	1.94 (0.25-15.02)	0.527	0	0	5	1
Tumor grade								
I	108	20	1.0		18	21	90	20
II	139	26	0.73 (0.36-1.48)	0.382	25	29	114	26
III	104	20	1.21 (0.62-2.37)	0.578	14	16	90	17
Estrogen receptor status								
Positive	444	84	1.0		71	83	373	84
Negative	82	15	2.62 (1.62-4.24)	0.0001	15	17	67	15
Progesterone receptor status								
Positive	359	68	1.0		62	72	297	69
Negative	168	32	2.04 (1.31-3.18)	0.002	23	28	144	32
p53 status								
Negative	262	49	1.0		44	51	218	49
Focal	151	28	0.99 (0.56-1.73)	0.966	19	22	132	30
Low	28	5	1.04 (0.37-2.94)	0.938	8	9	20	5
High	71	13	2.48 (1.44-4.27)	0.001	12	14	59	13
Age at Diagnosis								
<55	232	44	1.0		43	50	189	43
>55	298	56	0.76 (0.49-1.18)	0.220	43	50	254	57
Family history								
None	244	46	1.0		41	48	203	46
1° relative	128	24	0.87 (0.49-1.54)	0.627	20	23	108	24
2° relative	108	20	1.05 (0.59-1.86)	0.873	17	20	91	20
HER2/ <i>neu</i> status								
Negative	300	57	1.0		49	57	251	57
Focal	111	21	0.95 (0.54-1.67)	0.845	16	19	95	21
Low	63	12	0.81 (0.38-1.71)	0.576	10	13	53	12
High	50	9	1.19 (0.58-2.44)	0.629	9	10	41	9
Chemotherapy								
None	406	77	1.0		71	83	335	75
After surgery	118	22	1.91 (1.20-3.05)	0.007	15	17	103	23
Adjuvant therapy								
None	178	33	1.0		26	30	152	34
Radiation	220	42	1.11 (0.63-1.98)	0.713	44	51	176	40
Chemotherapy	30	6	3.25 (1.52-6.95)	0.002	1	1	29	7
Both	102	19	1.83 (0.99-3.38)	0.052	15	17	87	20
Tamoxifen								
Yes	250	47	1.0		45	52	205	46
No	280	53	0.71 (0.45-1.12)	0.143	41	48	239	54
Postmenopausal								
No	156	29	1.0		25	29	131	30
Yes	358	68	0.79 (0.49-1.26)	0.323	59	69	299	67
TC								
>200%	86	16	1.0		86	100	0	0
≤200%	444	84	3.14 (1.27-7.76)	0.013	0	0	444	100

NOTE: TNM stage was assigned using the 2002 revised criteria (20). Ethnicity and family history were based on self-report. See Materials and Methods for additional details.

of the protein and accumulation in tumor cell nuclei (24, 25). The cutoff levels for staining for p53 are negative (no staining), focal (<5% staining), low (5-39% staining), and high (40-100% staining). HER2/*neu* protein expression was evaluated using the anti-HER2/*neu* monoclonal antibody CB11 (Santa Cruz Biotechnology). The cutoff levels for staining for HER2/*neu* are negative (no staining observed or

membrane staining observed in <10% of tumor cells), focal (faint/barely perceptible membrane staining detected in >10% of tumor cells and cells only stained in part of their membrane), low (weak to moderate complete membrane staining observed in >10% of tumor cells), and high (moderate to strong complete membrane staining observed in >10% of tumor cells). The negative and focal groups are

considered clinically negative; whereas, the low and high groups are considered clinically positive.

Statistical methods. The distribution of risk factors in the high-TC and low-TC groups (Table 1) was evaluated by the Fischer's exact test. Missing data for each risk factor was evaluated categorically in the analysis, but these data were not reported. The associations between TC and both overall survival interval and breast cancer-free survival interval were evaluated using log-rank Kaplan-Meier survival analyses. Univariate and multivariate Cox proportional hazards analysis was used to compute the relative hazards for breast cancer-related adverse events (i.e., death due to breast cancer, breast cancer recurrence, or development of a new primary breast tumor), and the best overall model, defined as the lowest overall model fit P value, is reported. Covariate-adjusted estimates of the survival function by level of TC ($\leq 200\%$ versus $>200\%$) are the baseline survival estimates from a stratified proportional hazards model and were computed at the mean level of the covariates. Subjects were censored at the time lost to follow-up. P values of <0.05 were considered significant for all tests.

Results

TCs predict overall survival. To confirm prior associations observed between TC and overall survival interval, the cohort was initially divided into sixths, the survival interval for each group was calculated, and the results were evaluated for statistical significance by log-rank analysis. Groups with statistically indistinguishable survival intervals were combined, and the process was repeated until only groups with significantly different survival intervals remained. Using this process, the cohort was stratified into two TC groups: low TC was defined as $\leq 200\%$ of the placental DNA control ($n = 444$), and high TC was defined as $>200\%$ of TC in the placental DNA control ($n = 86$). Log-rank analysis showed a significant relationship between TC group and overall survival interval ($P = 0.025$), with low TC predicting a shorter survival interval.

The results are plotted by the method of Kaplan and Meier and shown in Fig. 1A. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.25 (95% CI, 1.09-4.64; $P = 0.029$) relative to high TC (not shown). The relationship between TC group and overall survival interval in the subset of invasive tumors (i.e., without the 97 ductal carcinoma *in situ* cases) was also evaluated. In this subset, log-rank analysis also showed a significant relationship between TC group and overall survival interval ($P = 0.046$). The results are plotted by the method of Kaplan and Meier and shown in Fig. 1B. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.06 (95% CI, 1.00-4.26; $P = 0.05$) relative to high TC (not shown).

TCs predict breast cancer-free survival. Next, we refined our criteria to evaluate the prognostic value of TC in predicting breast cancer-related, adverse event-free survival interval. An adverse event was defined as death due to breast cancer, breast cancer recurrence, or development of a new primary breast tumor. Seventy-nine breast cancer-related adverse events had occurred by the time of the analysis, including 46 deaths, 15 recurrences, and 18 new primary breast tumors. A Kaplan-Meier plot and log-rank test (Fig. 2A) showed significant differences in the groups' survival intervals ($P = 0.009$) with low TC, again predicting a shorter survival interval. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 3.14 (95% CI, 1.27-7.76; $P = 0.013$) relative to high TC (Table 1). The relationship between TC group and breast cancer-free survival in the subset of invasive tumors was also evaluated. In this subset, log-rank analysis also showed a significant relationship between TC group and breast cancer-free survival interval ($P = 0.032$). The results are plotted by the method of Kaplan and Meier and shown in Fig. 2B. A univariate Cox proportional hazards model showed low TC had

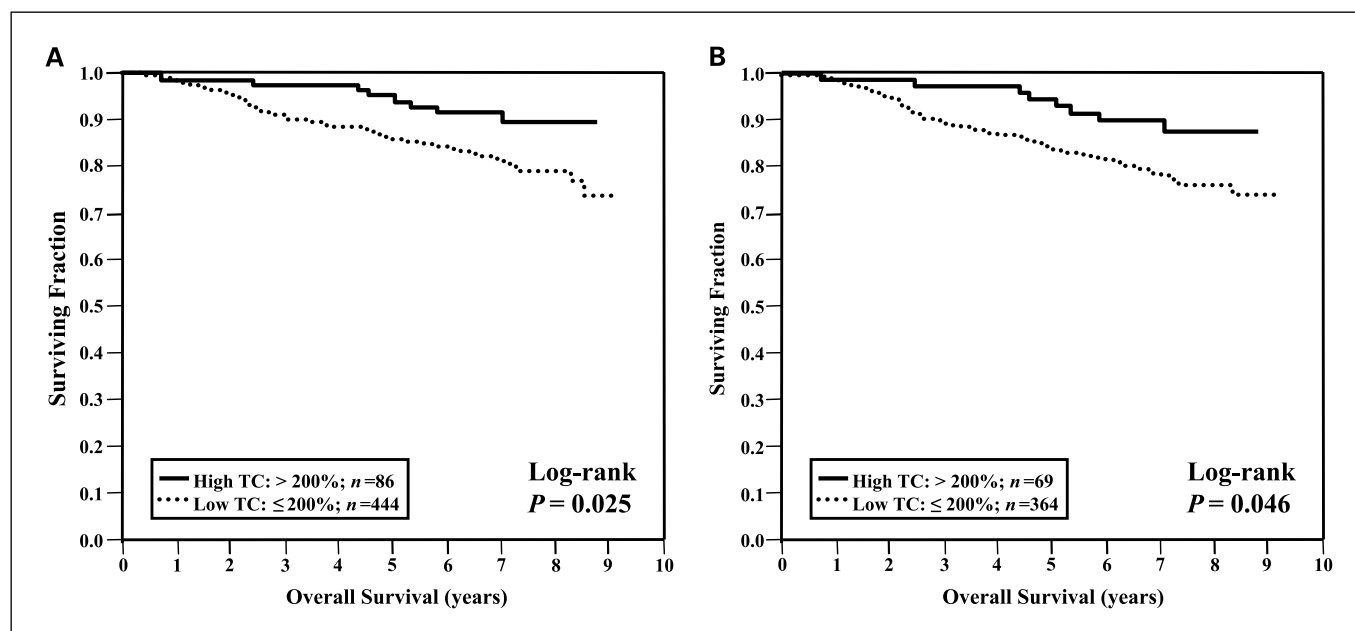


Fig. 1. Overall survival interval by TC in breast tumors. The set of all tumors (A) or invasive tumors only (B) was divided into two groups based on the low-TC and high-TC cutoff (200% of standard). Overall survival interval (in y) is shown on the x axis, and the surviving fraction is shown on the y axis. Subjects were censored at the time lost to follow-up. The log-rank test was used to test the significance (P) of the differences in the groups' survival intervals. n , number of subjects in each group.

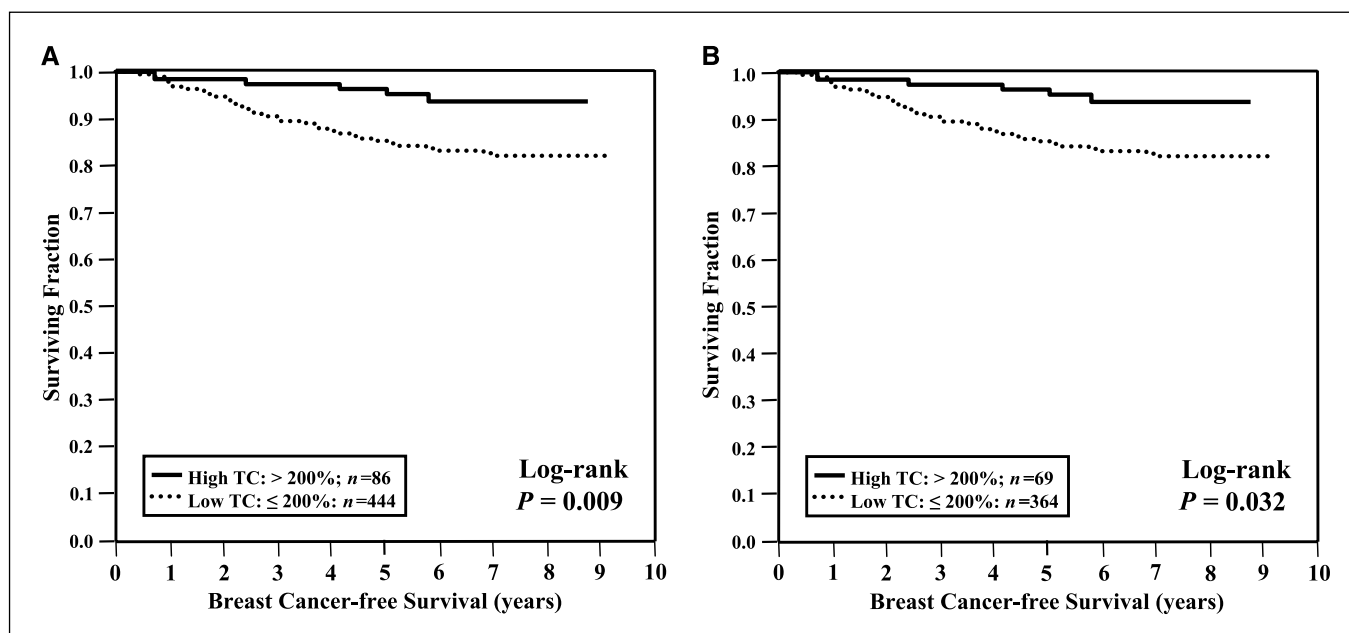


Fig. 2. Breast cancer-free survival interval by TC in breast tumors. The set of all tumors (A) or invasive tumors only (B) was divided into two groups based on the low-TC and high-TC cutoff (200% of standard). Breast cancer-free survival interval (in y) is shown on the x axis, and the recurrence-free fraction is shown on the y axis. See Fig. 1 for additional details.

an unadjusted relative hazard of 2.61 (95% CI, 1.05-6.48; $P = 0.039$) relative to high TC (not shown). Similarly, although not statistically significant, results were shown in the subset of ductal carcinoma *in situ* cases (not shown).

TC is an independent predictor of breast cancer-free survival. The relative hazards for breast cancer-related adverse events associated with 12 categorical risk factors were evaluated individually by Cox proportional hazards analysis (Table 1). Ethnicity, TNM stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy sequence, adjuvant therapy, and TC each conferred significant ($P < 0.05$) relative hazards. There was no significant hazard associated with age at diagnosis, family history of breast cancer, HER2/*neu*, or postmenopausal status or hormonal therapy. Pair-wise analysis using Fisher's exact test showed no significant difference in the distribution of any of the risk factors in the low-TC and high-TC groups (Table 1).

Multivariate Cox proportional hazards models were developed using TC and all combinations of the covariates that conferred significant relative hazards (ethnicity, TNM stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy sequence, adjuvant therapy, and TC). The best overall model (Table 2), defined as the lowest overall model fit P value, included TC, p53 and estrogen receptor status, and TNM stage ($P < 0.00005$). Relative to the high-TC group, low TC conferred an adjusted relative hazard of 2.88 (95% CI, 1.16-7.15; $P = 0.022$). The chemotherapy, adjuvant therapy, and hormonal therapy covariates were strongly associated with TNM stage and with each other ($P < 0.0001$). Therefore, additional multivariate Cox proportional hazards models were developed using TC and chemotherapy, adjuvant therapy, and hormonal therapy as covariates, either alone or in combinations. The best overall models, defined as the lowest overall model fit P value, included TC and either chemotherapy or adjuvant therapy

($P = 0.002$); the addition of the hormonal therapy covariate had no effect. In the second model, low TC conferred an adjusted relative hazard of 2.84 (95% CI, 1.14-7.05; $P = 0.025$), relative to the high-TC group (not shown).

Table 2. Relative hazards and 95% CIs from a multivariate Cox proportional hazards model of breast cancer-free survival interval from date of diagnosis of breast cancer

Variable	n	Level	Relative hazard (95% CI)	P
TC	86	>200%	1.00	
	444	≤200%	2.88 (1.16-7.15)	0.022
p53	441	None/focal/low	1.00	
	71	High	1.93 (1.10-3.38)	0.022
Estrogen receptor	444	Positive	1.00	
	82	Negative	1.69 (0.97-2.95)	0.063
TNM	259	I	1.00	
	97	0 (<i>in situ</i>)	0.98 (0.48-2.04)	0.967
	115	IIA	1.61 (0.90-2.88)	0.110
	46	IIB/IIIA	3.39 (1.81-6.36)	0.0001

NOTE: Multivariate Cox proportional hazards models of survival from date of diagnosis of breast cancer for breast cancer-free survival intervals were used to derive the adjusted relative hazards associated with each variable. Adjusted relative hazard values were developed using p53 status (none/focal/low versus high), TNM stage [I versus 0 (*in situ*) versus IIA versus IIB/IIIA], estrogen receptor status (present/absent), and TC group (≤200%/>200%) as independent predictors of survival. The 95% CIs for each relative hazard are shown in parenthesis. See Materials and Methods for additional details.

Discussion

TC is a convenient proxy for telomere length that is particularly well-suited for the analysis of samples where DNA is degraded or scant, such as sections from archival, FFPE tissues (15, 16). We used this method to determine TC values in tumor tissue collected in a prospective, population-based cohort composed of 530 women and evaluated the associations of TC with clinical variables and end points, including overall and breast cancer-free survival intervals.

The principal conclusion from this investigation is that TC predicts breast cancer-free survival interval, independent of 12 clinical factors, prognostic markers, and adjuvant therapies. Tumors with TC of $\leq 200\%$ of placental DNA standard conferred an adjusted hazard for breast cancer recurrence of 2.88 (95% CI, 1.16-7.15; $P = 0.022$). These results, obtained from a large population-based cohort, are in accord with our recent study (17) of breast tumors (predominantly TNM stage IIA and above) that also showed highly significant associations between TC and overall 5-year survival ($P < 0.0001$) and breast cancer-free survival interval (relative hazard, 4.43; 95% CI, 1.44-13.64; $P = 0.009$). Likewise, our previous investigation of prostate cancer (18) revealed that TC was also associated with time to prostate cancer recurrence (relative hazard, 5.02; 95% CI, 1.40-17.96; $P = 0.013$), controlling for age at diagnosis, Gleason sum, and pelvic node involvement. Similar results were obtained when analyses were done using the subset of invasive tumors, and a similar trend was observed in the subset of ductal carcinoma *in situ* cases. These data suggest that TC may be able to predict clinical outcome in both invasive tumors and ductal carcinoma *in situ* cases. As discussed above, adjuvant polychemotherapy in node-negative patients with ages < 50 years improves 10-year survival from 71% to 78% (a 24% increase, i.e., seven per 29%), whereas in patients with ages 50 to 70 years, adjuvant therapy improves 10-year survival from 67% to only 69% (a 6% increase, i.e., two per 33%). A TC threshold of $> 200\%$ of the standard defines a subgroup comprising of $\sim 17\%$ of the population-based cohort that have a significantly reduced risk of disease recurrence (7% at 8 years) that would be potential candidates for less aggressive adjuvant therapy. However, subsequent experiments in larger cohorts are needed to extend these findings.

The point estimate of the relative hazard for breast cancer recurrence associated with "low" TC was lower than in our prior investigation (2.88 versus 4.43), although the confidence intervals overlap. One possibility is that the discrepancy in the point estimates reflects the difference in the length of follow-up in the two studies. The mean, maximum, and interquartile ranges for follow-up in the HEAL cohort were 6.7, 9.2, and 1.5 years, respectively, versus 9.1, 23, and 11.2 years, respectively, in the prior study (17). The ongoing follow-up of the HEAL cohort will resolve this question. It is also important to consider that HEAL is a prospective study in which FFPE tissue samples were collected for participants at

multiple independent sites at the time of diagnosis before the start of follow-up, rather than a retrospective study of archival tissues from a single facility, which is more open to inadvertent selection bias.

Another important difference between these two studies is that the TC threshold used to discriminate women at risk for breast cancer recurrence, $> 123\%$ and $> 200\%$ in the prior and present studies, respectively. This difference may also reflect the differences in the lengths of follow-up, in which case we would expect that the threshold will decrease as more deaths and adverse effects occur. Alternatively, the discrepancies in threshold, as well as the point estimates for the relative hazard ratios, could reflect either the larger number of specimens (530 versus 77) or the larger fraction of localized tumors (stages 0 and I) in the HEAL cohort and prior cohort (67% versus 14%).

Here, using the HEAL cohort, we have shown that TC predicts breast cancer-free survival interval independent of other risk factors. It is important to note that these other established risk factors, such as ethnicity, TNM stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy, and adjuvant therapy also conferred significant univariate relative hazards for breast cancer-related adverse events, confirming a representative population cohort. However, this population was not selected for TC (or any other biomarker) analysis and, thus, represents an unbiased assessment of TC as a prognostic factor. Telomere shortening has been associated with age in normal tissues (26); however, in this study, there was no association between TC and patient age, which is consistent with our previous results (17, 18). This indicates that telomere attrition due to tumorigenesis far exceeds the shortening contributed to age alone. Additionally, it must be noted that the cutoff established in this study, $> 200\%$ of the placental DNA standard, exceeds the 95% CI for TC in several normal tissues (75-143% of standard), including breast (17). Speculatively, these longer telomeres may result from the early up-regulation of telomerase during tumor progression.

In summary, TC in tissues from breast tumors is an independent predictor in this group of breast cancer-free survival interval. In the future, TC, in combination with extant prognostic markers, could provide women and their physicians new information to guide therapeutic decisions. However, the assay in its current format, due to the relatively complex experimental procedure, is more suitable for use in a research rather than clinical setting. Therefore, development of a platform for TC determination that is simple and readily adaptable to a clinical laboratory is necessary before these findings can be validated in independent laboratories with independent cohorts.

Acknowledgments

We thank William Hunt and Sharon Wayne for their help with the statistical analysis and Dr. Melanie Royce for her critical evaluation of the manuscript.

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Assessment of the Frequency of Allelic Imbalance in Human Tissue Using a Multiplex Polymerase Chain Reaction System

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Genomic instability can generate chromosome breakage and fusion randomly throughout the genome, frequently resulting in allelic imbalance, a deviation from the normal 1:1 ratio of maternal and paternal alleles. Allelic imbalance reflects the karyotypic complexity of the cancer genome. Therefore, it is reasonable to speculate that tissues with more sites of allelic imbalance have a greater likelihood of having disruption of any of the numerous critical genes that cause a cancerous phenotype and thus may have diagnostic or prognostic significance. For this reason, it is desirable to develop a robust method to assess the frequency of allelic imbalance in any tissue. To address this need, we designed an economical and high-throughput method, based on the Applied Biosystems AmpF/STR Identifiler multiplex polymerase chain reaction system, to evaluate allelic imbalance at 16 unlinked, microsatellite loci located throughout the genome. This method provides a quantitative comparison of the extent of allelic imbalance between samples that can be applied to a variety of frozen and archival tissues. The method does not require matched normal tissue, requires little DNA (the equivalent of ~150 cells) and uses commercially available reagents, instrumentation, and analysis software. Greater than 99% of tissue specimens with ≥ 2 unbalanced loci were cancerous. (*J Mol Diagn* 2007, 9:266–271; DOI: 10.2353/jmoldx.2007.060115)

It is widely accepted that genomic instability—the duplication, loss, or structural rearrangement of a critical gene(s)—occurs in virtually all cancers¹ and in some instances has diagnostic, prognostic, or predictive significance. Thus, it is not surprising that tumor progression is reflected by allelic losses or gains in genes that regu-

late aspects of cell proliferation, apoptosis, angiogenesis, invasion, and, ultimately, metastasis.^{2,3}

There are several technologies available to detect chromosomal copy number changes in tumor cells. For example, chromosome painting techniques can identify chromosomal copy number changes in cytological preparations.^{4,5} Segmental genomic alterations can be identified by comparative genomic hybridization (CGH). CGH identifies copy number changes by detecting DNA sequence copy variations throughout the entire genome and mapping them onto a cytogenetic map supplied by metaphase chromosomes.⁶ On the other hand, array CGH maps copy number aberrations relative to the genome sequence by using arrays of bacterial artificial chromosome or cDNA clones as the hybridization target instead of the metaphase chromosomes.^{7–11} However, these methods cannot identify all cases of allelic imbalance (AI), which is a deviation from the normal 1:1 ratio of maternal and paternal alleles, for instance, in cases with uniparental disomy. In addition, these methods are poorly suited for high-throughput applications, and analysis is limited to a relatively small cellular field, thus increasing potential sampling error.

Single nucleotide polymorphism (SNP) arrays can be used for high-resolution genome-wide genotyping and loss of heterozygosity detection.^{12–14} For example, the development of a panel of 52 microsatellite markers that detects genomic patterns of loss of heterozygosity^{15–17} has been used for breast cancer diagnosis and prognosis. However, this approach requires matched referent (normal) DNA, typically blood or buccal samples, and these cancer-type-specific panels may not be informative for other cancers, thus limiting their applicability across multiple tumor types. Larger panels of SNPs may be used for genome-wide analysis, for example, the Affymetrix 10K and 100K SNP mapping arrays.^{18,19} Likewise, Illumina BeadArrays with a SNP linkage-mapping panel²⁰ allow allelic discrimination directly on short genomic seg-

Supported by the Department of Defense (Breast Cancer Research Program: DAMD17-02-1-0514, W81XWH-05-1-0273, and W81XWH-04-1-0370; Prostate Cancer Research Program: W81XWH-04-1-0831 and W81XWH-06-1-0120) and the National Institutes of Health (grant RR0164880).

Accepted for publication November 17, 2006.

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ments surrounding the SNPs of interest, thus overcoming the need for high-quality DNA.¹⁴ Lips and colleagues²¹ have shown that Illumina BeadArrays can be used to obtain reliable genotyping and genome-wide loss of heterozygosity profiles from formalin-fixed, paraffin-embedded (FFPE) normal and tumor tissues. However, all these approaches, although robust, require costly reagents and specialized equipment, and the sheer amount of data produced from these analyses complicate the interpretation of results.

For these reasons, and as outlined by Davies and colleagues,²² it is desirable to develop a general, economical, and high-throughput method to assess the frequency of AI in any tissue, independent of the nature and composition of the specimen and the availability of matched, normal tissue. To address this need, we developed a method to measure the ratio of maternal and paternal alleles at 16 unlinked, microsatellite short tandem repeat (STR) loci in a single multiplexed polymerase chain reaction (PCR). The assay, which is based on the Applied Biosystems AmpF/STR Identifier system, can be performed with only 1 ng of genomic DNA and uses commercially available primers and reagents as well as common instrumentation and analysis software. Thus, it is an attractive alternative to current methods and is readily adaptable to most clinical laboratory environments.

Materials and Methods

Tissue Acquisition

All tissues were provided by the University of New Mexico Solid Tumor Facility, unless otherwise specified. Buccal cells were collected from oral rinses of volunteers. The Cooperative Human Tissue Network (Western Division, Nashville, TN) provided frozen normal and tumor renal tissues obtained by radical nephrectomy, frozen normal breast tissues obtained by reduction mammoplasty, and normal frozen prostate tissues obtained through autopsy. A set of FFPE prostate tumors obtained by radical prostatectomy was provided by the Cooperative Prostate Cancer Tissue Resource (<http://www.cpctr.cancer.gov>). Duodenal FFPE tumor tissues were obtained from the Mayo Clinic (Rochester, MN). Pancreatic FFPE normal and tumor tissues were obtained from the Department of Pathology at the University of New Mexico. Frozen endometrial tumor tissues were obtained through the Gynecologic Oncology Group (Philadelphia, PA). All specimens lacked patient identifiers and were obtained in accordance with all federal guidelines, as approved by the University of New Mexico Human Research Review Committee.

DNA Isolation and Quantification

DNA was isolated from all tissue samples using the DNeasy silica-based spin column extraction kit (Qiagen, Valencia, CA) and the manufacturer's suggested animal tissue protocol. FFPE samples were treated with xylene and washed with ethanol before DNA extraction. DNA

concentrations were measured using the Picogreen dsDNA quantitation assay (Molecular Probes, Eugene, OR) using a λ phage DNA as the standard as directed by the manufacturer's protocol.

Multiplex PCR Amplification of STR Loci

The AmpF/STR Identifier kit (Applied Biosystems, Foster City, CA) was used to amplify genomic DNA at 16 different STR microsatellite loci (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX, and vWA) in a single multiplexed PCR reaction, according to the supplier's protocol. Linear amplification of allelic PCR products is a prerequisite for ratiometric determination of AI. Therefore, each PCR reaction was limited to 28 cycles, as determined in preliminary studies. The 16 primer sets are designed and labeled with either 6-FAM, PET, VIC, or NED to permit the discrimination of all amplicons in a single electrophoretic separation. The PCR products were resolved by capillary electrophoresis using an ABI Prism 377 DNA sequencer (Applied Biosystems). Fluorescent peak heights were quantified using ABI Prism GeneScan analysis software (Applied Biosystems). Allelic ratios were calculated using the peak height, rather than the peak area, as suggested in previous studies.²³⁻²⁵ For simplicity, the allele with the greater fluorescence was always made the numerator, as to always generate a ratio ≥ 1.0 .

Statistical Analysis

A Pearson χ^2 test was performed using SAS JMP software version 9.1 (SAS Institute Inc., Cary, NC) to examine the relationship between the extent of AI and tissue type, using a significance level of 0.05.

Results

The 16 allelic microsatellite loci amplified by the AmpF/STR Identifier primer sets are unlinked and can be used to assess AI simultaneously at multiple heterozygous sites throughout the genome. This is technically possible because each amplicon is labeled with one of four fluorescent dyes (6-FAM, PET, VIC, and NED), each with a unique emission profile, thus allowing the resolution of amplicons of similar size. Figure 1 shows the sizes of VIC-labeled amplicons derived from a representative specimen of matched normal and tumor renal tissue (the fluorescent channels showing the PET-, 6-FAM-, and NED-labeled products are not shown). Within Figure 1A, illustrating the results from the normal tissue specimen, two of the allelic pairs are homozygous (D13S317, D16S539), as indicated by a single peak, and three of the allelic pairs are heterozygous (D3S1358, TH01, D2S1338), as indicated by two peaks. Although the peak heights varied between different loci, ostensibly because of different PCR efficiencies, the peak heights of the paired alleles were similar. Theoretically, the ratio of any

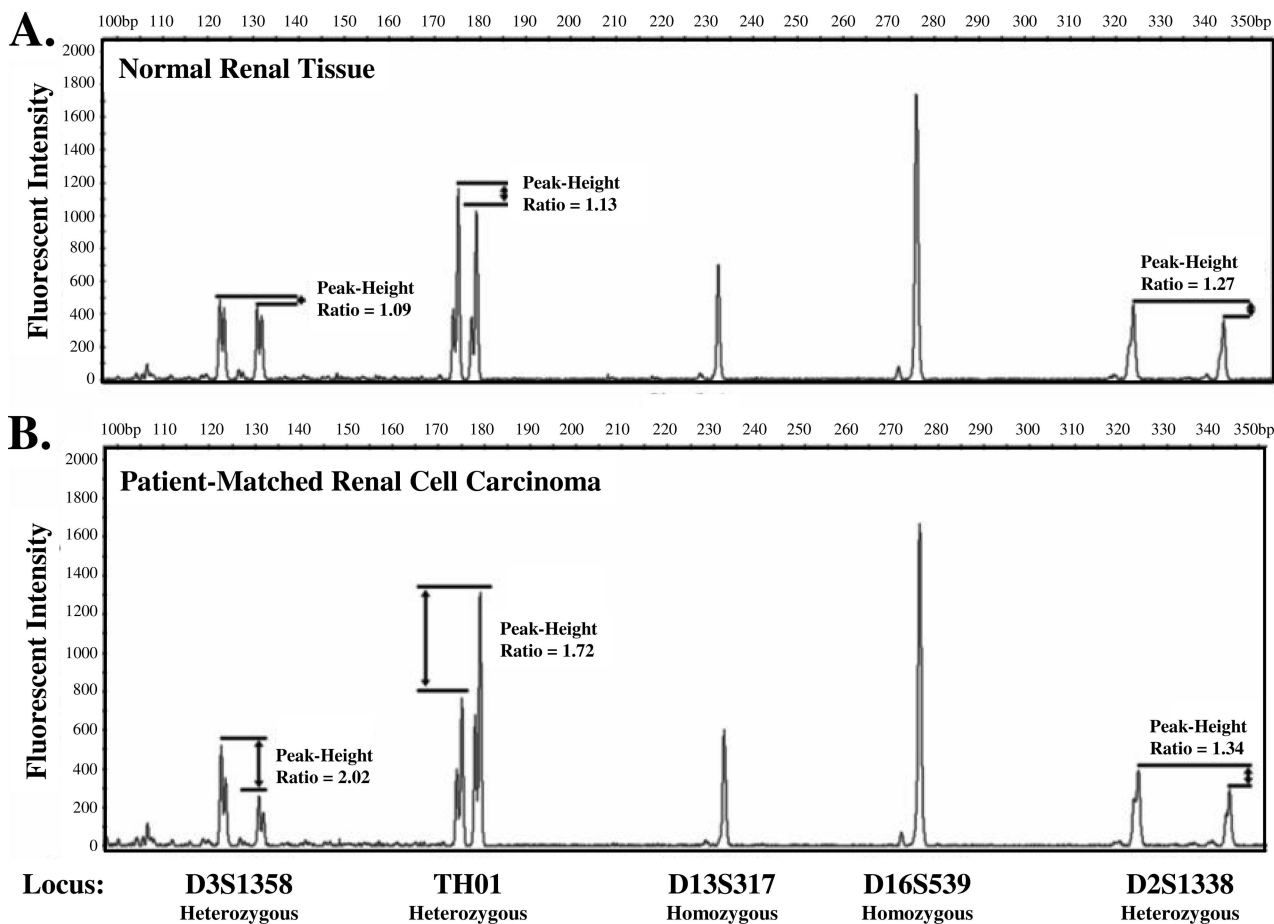


Figure 1. Electropherograms of VIC-labeled amplicons from a matched normal and renal carcinoma sample. PCR was performed and the resulting amplicons resolved as described in Materials and Methods. Only VIC-labeled amplicons are shown. In this particular sample, the D3S1358, THO1, and D2S1338 loci are heterozygous, and D13S317 and D16S539 loci are homozygous. Fluorescence intensity is shown on the y axis, and amplicon size, in bp, is shown on the x axis. The ratios of the fluorescent intensities of each allelic pair of heterozygous loci are shown. Loci with allelic ratios of >1.60 are defined as sites of AI for matched normal (A) or tumor (B) tissue.

two heterozygous alleles is 1.0 in normal tissues. However, differences in PCR efficiency between different length alleles and random experimental variation resulting from instruments, reagents, and personnel may affect the observed ratio of heterozygous alleles. To assess these sources of potential variation, the ratios of paired alleles' signal intensities were compared at 320 heterozygous loci in buccal cells from 27 healthy individuals. Across all loci, the mean ratio was near 1.0 (mean, 1.15; SD, 0.18). We expect that $\sim 97.5\%$ of all allelic ratios in normal tissues would fall within 2.5 SD of the mean and therefore operationally defined an allelic ratio of >1.60 (mean +2.5 SD) as a site of AI. Applying this threshold to the 27 analyzed buccal samples, only eight sites of AI were detected of the 320 heterozygous loci, thus representing a mean of 0.30 unbalanced loci per sample. Figure 1B illustrates the results of the tumor tissue matched to the normal sample in Figure 1A. Within this sample, two of the three heterozygous loci in the renal tumor tissue amplified by the VIC-labeled primer sets have peak height ratios of >1.60 , identifying them as sites of AI.

To determine whether AI determinations were reproducible, the assay was repeated within a random subset

of the buccal samples. The mean absolute variation of the allelic ratios for the repeated samples was 10% and 193 of the 198 (97.5%) loci measured were correctly categorized on repeating the experiment; however, only five of the 198 (2.5%) loci initially designated as sites of AI could not be confirmed (Figure 2A). Two loci changed from sites without AI (≤ 1.60) to sites of AI (>1.60), and three loci changed from sites of AI to sites without AI.

We next confirmed that the differences in AI detected by this approach reflected true differences in the ratio of the alleles, not experimental artifact (eg, differential PCR amplification efficiency). We constructed defined mixtures of DNAs from the paired normal and tumor tissue shown in Figure 1. As shown in Figure 2B for the D3S1358 locus, there was a linear relationship ($R^2 = 0.965$) between the ratio of alleles measured in the assay and the composition of the mixture. Similar results were obtained for each of the other loci exhibiting a site of AI (THO1, $R^2 = 0.973$; VWA, $R^2 = 0.981$; D18S541, $R^2 = 0.953$). In contrast, the composition of the mixture had no effect on the allelic ratios of loci not exhibiting AI (data not shown).

The operationally defined threshold for AI was validated by measuring the allelic ratios for 1382 heterozygous loci in an independent test set comprised of 118

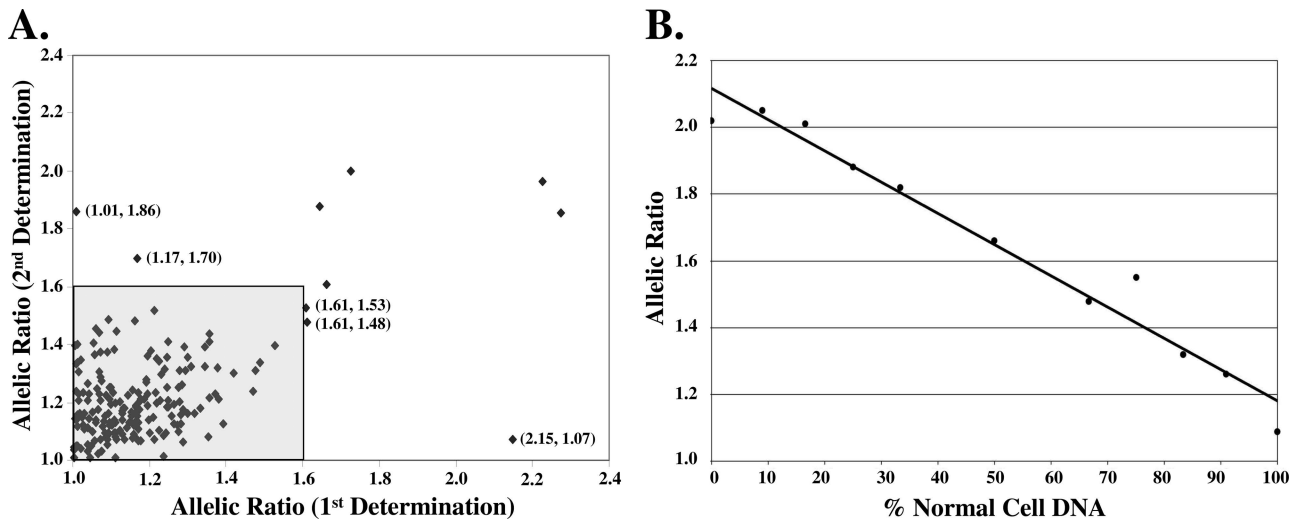


Figure 2. Reproducibility and effect of admixtures of matched normal and renal carcinoma DNA on allelic peak height ratios. **A:** Allelic peak height ratios were determined for 198 heterozygous loci in 16 normal buccal samples. The plot represents the first determination (*x* axis) and the second determination (*y* axis). The region defined by the **gray shaded box** represents all of the loci that were determined not to be a site of AI on both determinations. The labeled points (allelic peak height ratios for both determinations) represent the five loci that were not correctly identified on repeating the experiment. **B:** The specified admixtures were generated using DNA from a matched pair of normal renal tissue and renal cell carcinoma as shown in Figure 1. Data from the heterozygous D3S1358 locus are shown. The allelic ratios are 1.09 in the normal renal tissue and 2.02 in the renal carcinoma. The best-fit line was generated by linear regression and has a correlation coefficient (R^2) of 0.965.

normal samples consisting of bone ($n = 2$), breast ($n = 10$), buccal ($n = 53$), lymph node ($n = 5$), peripheral blood lymphocytes ($n = 18$), pancreas ($n = 6$), placenta ($n = 3$), prostate ($n = 4$), renal ($n = 16$), and tonsil ($n = 1$) tissues (Figure 3A). In this sample set of normal tissues, only 32 of 1382 heterozygous loci were designated sites of AI, thus representing a mean of 0.27 unbalanced

loci per sample, comparable with the 0.30 unbalanced loci per sample in the original normal sample set. In summary, 88 (74.6%), 29 (24.6%), and one (0.8%) of the 118 normal tissues specimens contained zero, one, and two loci with AI, respectively.

It is well established that cancerous tissues have more sites of AI than normal tissues. To validate our assay in this context, we next measured the frequency of AI in 2792 heterozygous loci in a set of 239 frozen or FFPE tumor samples consisting of acute myelogenous leukemia ($n = 8$), breast ($n = 39$), chronic myelogenous leukemia ($n = 3$), duodenal ($n = 23$), endometrial ($n = 78$), pancreas ($n = 6$), prostate ($n = 47$), and renal ($n = 35$) tissues. As shown in Figure 3B, 37 (15.5%), 41 (17.2%), and 161 (67.4%) of the 239 tumor tissues specimens contained zero, one, and more than or equal to two loci with AI, respectively. In contrast to the normal tissues, 611 sites of AI were detected, thus representing a mean of 2.56 unbalanced loci per sample, nearly 10 times greater than the frequency in the normal tissues ($P < 0.0001$). In summary, 162 of 357 tissue specimens had ≥ 2 unbalanced loci, of which $>99\%$ were cancerous.

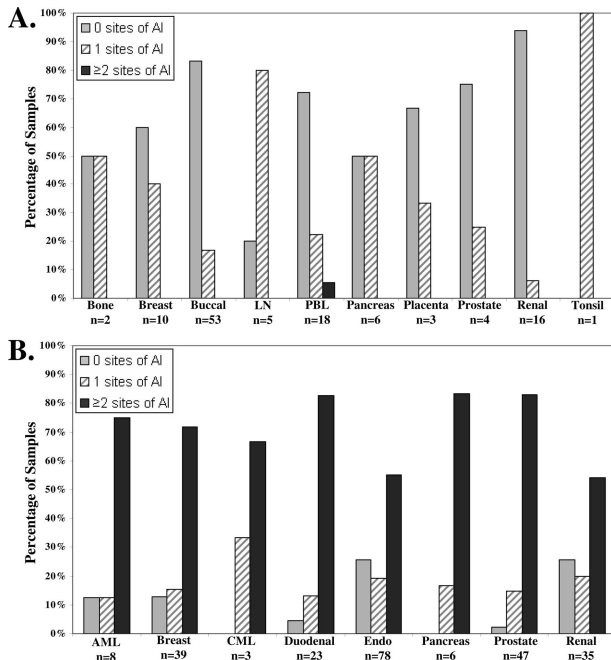


Figure 3. Frequency of AI in normal and tumor tissues. The numbers of sites of AI (ie, 0, 1, ≥ 2) were determined in 118 samples of normal tissue (**A**) and in 239 samples of tumor tissue (**B**). The number of specimens in each tissue set (n) is indicated below the set designation. LN, lymph node; PBL, peripheral blood lymphocytes; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; Endo, endometrial. See Materials and Methods for additional details.

Discussion

The frequency of AI reflects the karyotypic complexity of the cancer genome and such manifestations are widespread in solid tumors.¹ There have been numerous studies of these abnormalities and several techniques, including chromosome painting, array CGH, and SNP arrays, have emerged to analyze these differences between normal and tumor tissues.^{4–21} However, these methods are typically costly, time intensive, and need a matched referent (normal) DNA sample for analysis. For this reason,

it is desirable to develop general, economical, high-throughput methods to quantify the extent of AI in the genome of any tissue, independent of the nature and composition of the specimen and the availability of matched, normal tissue.

Using our newly developed assay and interpretation scheme to assess the frequency of AI in human tissues, we have shown in a set of 239 samples that 67% of the tumors contained two or more sites of AI, as compared with 0.8% of the normal samples, which represents an almost 84-fold difference. It must be noted that tissue heterogeneity, such as a preponderance of normal cells within the tumor, may quench peak-height ratios below the 1.60 threshold, thus obscuring AI in a particular sample. In addition, the assay cannot discriminate between homozygous alleles and complete loss of heterozygosity in the absence of matched normal tissue. However, the latter limitation is mitigated by the near ubiquitous presence of normal tissue within tumors, which allows for the assessment of AI in samples without requiring analysis of matched normal tissue. This is an important consideration in the potential evaluation of biopsy tissue, which may contain multiple clones of genetically altered cells superimposed on a background of normal stromal and epithelial cells, and obtaining matched normal tissue may be difficult.

Altered gene expression resulting from genomic instability is a cause of cancer progression; therefore, cancerous tissues have more sites of AI than normal tissues. Consistent with this observation, >99% of tissues with ≥ 2 sites of AI were cancerous. We are currently investigating the possibility that the number of sites of AI in cancer tissue is a reflection of its stage of progression and therefore may correlate with clinical parameters or prognosis.

Existing alternative methods identify AI as a difference in the allelic ratios in the sample of interest (eg, tumor) relative to the allelic ratios in a patient-matched referent DNA. These methods allow for the distinction between complete loss of heterozygosity and a constitutive homozygous allele and are able to control for PCR efficiency differences of alleles of dissimilar length. In contrast, the present method identifies AI as a deviation from a 1:1 ratio between alleles within the sample of interest only. Thus, the assay described herein can be performed on specimens for which a reliable referent sample is not available. In addition, we have determined that the mean absolute variation of the allelic ratios for all microsatellite loci in our panel is $\sim 15\%$ in normal tissues. This variation represents the combined effects of 1) random experimental error resulting from instruments, reagents, and personnel; 2) copy number polymorphisms; and 3) inherent differences in the PCR efficiencies of microsatellite alleles of dissimilar lengths. Based on replicate experiments of the same sample (Figure 2A), we have determined that random experimental variation resulting from instruments, reagents, and personnel accounts for $\sim 10\%$ of the overall variation. Therefore, variation resulting from differences in PCR efficiencies is $\sim 5\%$. Although the latter variation is excluded by comparison to a referent DNA, the requirement for two determinations (sample of interest and referent), each with an average variation of at

least 10%, minimizes the benefit gained by controlling for PCR efficiency.

In conclusion, we describe here a simple method for assessing the extent of AI throughout the genome. This method has a number of significant advantages over existing technologies, such as chromosome painting, array CGH, and SNP arrays and, as a molecular-based assay, may be used clinically in conjunction with histological techniques. The advantages of this method are that 1) it is robust, reproducible, and provides a quantitative basis for comparing the extent of AI between samples; 2) it does not require matched normal tissue; 3) it utilizes commercially available reagents, instrumentation, and analysis software; 4) it can be applied to a variety of fresh, frozen, and archival tissues; 5) it requires very little DNA (the equivalent of ~ 150 cells); and 6) >99% of tissues with ≥ 2 sites of AI were cancerous.

Acknowledgments

We thank Terry Mulcahy and Phillip Enriquez III, from the DNA Research Services of the University of New Mexico Health Sciences Center for gel capillary analysis; and Dr. Artemis Chakerian from the University of New Mexico Experimental Pathology Laboratory for tissue sectioning.

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Genomic instability demonstrates similarity between DCIS and invasive carcinomas

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Received: 18 June 2008 / Accepted: 14 August 2008
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Abstract *Purpose* To assess telomere DNA content (TC) and the number of sites of allelic imbalance (AI) as a function of breast cancer progression. *Experimental design* TC and AI were determined in 54 histologically normal tissues, 10 atypical ductal hyperplasias (ADH), 122 in situ ductal carcinomas (DCIS) and 535 invasive carcinomas (Stage I–IIIa). *Results* TC was altered in ADH lesions (20%), DCIS specimens (53%) and invasive carcinomas (51%). The mean number of sites of AI was 0.26 in histologically normal group tissue, increased to 1.00 in ADH, 2.94 in DCIS, and 3.07 in invasive carcinomas. All groups were statistically different from the histologically normal group ($P < 0.001$ for each);

however, there was no difference between DCIS and the invasive groups. *Conclusions* Genomic instability increases in ADH and plateaus in DCIS without further increase in the invasive carcinomas, supporting the notion that invasive carcinomas evolve from or in parallel with DCIS.

Keywords Allelic imbalance · Breast cancer · Ductal carcinoma in situ · Genomic instability · Telomere DNA content

Introduction

It is widely accepted that genomic instability is a prerequisite for the initiation and progression of virtually all cancers [1]. Accordingly, the progression of breast cancer can be characterized by the accumulation of genetic mutations in critical genes accompanied by histological progression from normal epithelium to atypical ductal hyperplasia (ADH), to ductal carcinoma in situ (DCIS) to the development of an invasive breast carcinoma [2, 3].

A significant cause of genomic instability is telomere dysfunction [4–7]. Telomeres are nucleoprotein complexes that are comprised of 1,000–2,000 tandemly repeated copies of the hexanucleotide DNA sequence (TTAGGG) [8]. These repeat regions are associated with numerous telomere binding proteins, such as Telomeric Repeat-binding Factor 1 (TRF1), Telomeric Repeat-binding Factor 2 (TRF2) and Protection of Telomeres 1 (POT1), which play important roles in telomere maintenance [9, 10]. Telomeres are located at and stabilize the ends of eukaryotic chromosomes, thus preventing degradation and recombination [11–13]. However, telomeres can be critically shortened, and thereby become dysfunctional, by several mechanisms, including incomplete replication of the lagging strand during DNA

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synthesis [14], loss or alterations of the telomere-binding proteins involved in telomere maintenance [15], and DNA damage induced by oxidative stress [16]. Telomere loss may be compensated by the reactivation of the enzyme telomerase, as seen in 85–90% of human cancers [17].

Abnormalities in telomere length are early and frequent events in the malignant transformation of numerous types of carcinomas [18, 19]. In breast, telomere shortening has been observed in invasive carcinomas, in situ lesions, and histologically normal tissue proximal to breast tumors [20, 21]. Additionally, our laboratory has recently demonstrated that telomere DNA content (TC), a proxy for telomere length, in breast tumor tissues is a prognostic marker for clinical outcome [22, 23].

Genomic instability can also be manifested by the presence of allelic imbalance (AI), which is a deviation from the normal 1:1 ratio of maternal and paternal alleles. Numerous studies have shown that the presence of AI is characteristic of invasive breast carcinomas [24, 25] and is also present at the in situ stage of the disease [26, 27]. Additional studies have demonstrated that AI occurs within atypical breast hyperplasias [28, 29], histologically normal tissue proximal to breast tumors [21, 30–32], and, in some instances, breast tissue from women with benign breast disease [33]. AI has also been found in the stromal compartment of cancer-associated breast tissues [34].

Numerous groups have investigated AI in the development of breast cancer. Notably, Ellsworth et al. [35] developed a panel of microsatellite markers specific for loci commonly lost in breast cancer. This group examined the evolution of genomic instability by characterizing AI in tissue samples representing a continuum of breast cancer development and concluded that DCIS lesions contain AI levels characteristic of advanced invasive tumors [36].

To evaluate the link between telomere dysfunction and the generation of allelic imbalance in the progression of breast cancer, we assessed alterations in TC and the extent of AI in a continuum of breast tissues ranging from histologically normal tissue derived from reduction mammoplasty, to ADH, DCIS and invasive carcinomas ranging from Stage I to IIIA. Here, we demonstrate that genomic instability (i.e. changes in TC or AI that exceed values typically observed in normal tissues) increases along the continuum of breast disease; however, it plateaus in DCIS without further increase in the invasive carcinomas.

Materials and methods

Tissue samples

A total of 721 human breast tissues were used in this study. Fifty-four normal, disease-free breast tissue

samples from women undergoing reduction mammoplasty (mean age = 35.6 years; range: 17–68) were obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN). Ten atypical ductal hyperplasia lesions (mean age = 56.3 years; range: 41–70) were obtained from the Department of Pathology at University of New Mexico Hospital (UNMH). Two independent cohorts of breast tumors were analyzed. The first cohort (test set) was obtained through the New Mexico Tumor Registry (NMTR) and Department of Pathology at UNMH and consisted of 163 specimens including DCIS ($N = 27$), and Stage I ($N = 104$) and IIA ($N = 32$) invasive breast carcinomas (mean age = 47.5 years; range: 25–77). The second cohort (validation set) was obtained through the Health, Eating, Activity and Lifestyle (HEAL) Study, an ongoing population-based, multi-center prospective cohort study [37], and consisted of 494 cases including DCIS ($N = 95$), and Stage I ($N = 244$), IIA ($N = 112$), Stage IIB ($N = 39$) and IIIA ($N = 4$) invasive breast carcinomas (mean age = 59.3 years; range: 29–89). Clinical data for the two tumor cohorts are shown in Table 1. Experiments were performed in accordance with all federal guidelines as approved by the University of New Mexico Health Science Center Human Research Review Committee.

Histological review

All tissue sections were examined microscopically to confirm diagnosis. Tissue sections were not microdissected, but typically contained from 75 to 100% tumor cells. A single pathologist reviewed the histological slides for the 10 ADH lesions and cohort two (validation set); whereas, the reduction mammoplasty specimens and cohort one (test set) were reviewed by numerous pathologists. The criteria used for the ADH specimens were based on morphological characteristics of a proliferative lesion that fulfills some but not all the criteria for DCIS.

DNA isolation and quantification

DNA was isolated from fresh, frozen or formalin-fixed, paraffin-embedded (FFPE) tissue samples using the DNeasy[®] silica-based spin column extraction kit (Qiagen; Valencia, CA) and the manufacturer's suggested animal tissue protocol. FFPE samples were treated with xylene and washed with ethanol prior to DNA extraction. DNA concentrations were measured using the Picogreen[®] dsDNA quantitation assay (Molecular Probes, Eugene, OR) using a λ phage DNA as the standard as directed by the manufacturer's protocol.

Table 1 Clinical and pathological characteristics of the analyzed breast tumors

Characteristic	Cohort #1 (N = 163)		Cohort #2 (N = 494)	
	N	%	N	%
<i>Ethnicity</i>				
NHW	106	65	380	77
Hispanic	27	17	114	23
Unknown	30	18	0	0
<i>TNM stage</i>				
0 (in situ)	27	16	95	19
I	104	64	244	49
IIA	32	20	112	23
IIB	0	0	39	8
IIIA	0	0	4	1
<i>Node status</i>				
Negative	163	100	261	53
Positive	0	0	107	22
Unknown	0	0	126	25
<i>ER status</i>				
Positive	80	49	418	85
Negative	46	28	72	14
Unknown	37	23	4	1
<i>PR status</i>				
Positive	72	44	340	69
Negative	53	33	151	30
Unknown	38	23	3	1
<i>Age</i>				
Mean	47.5		59.3	
Range	25–77		29–89	

TNM stage was assigned using the 2002 AJCC revised criteria. Ethnicity was self-reported. N, Number of specimens; ER, estrogen receptor; PR, progesterone receptor; NHW, non-Hispanic White. For additional details, see Materials and Methods Section

Telomere DNA content (TC) assay

TC was measured in known DNA masses, typically 5–10 ng, by slot blot titration assay, as previously described [21–23]. TC is expressed as a percentage of the TC in a placental DNA standard measured in parallel, which is defined as 100%. Each measurement was repeated independently three times and the coefficient of variation for each sample was $\leq 10\%$. The content of telomere DNA sequences can easily be measured in genomic DNA obtained from fresh, frozen and paraffin-embedded tissues [22, 38]. We have previously shown that TC is (i) directly proportional to telomere length determined by Southern blotting, (ii) not affected by TTAGGG sequences outside the telomere, and (iii) not affected by DNA fragmentation less than 1 KB in length [22, 38].

Determination of allelic imbalance

The extent of AI was determined using a straight-forward, economical, and high-throughput method recently developed by our laboratory [39]. This method evaluates AI in a panel of 16 randomly selected microsatellite markers (i.e. markers with no known relationship to breast cancer) thereby preventing measurement bias by selection of genes whose products are involved in tumorigenesis [39]. Briefly, DNA (~ 1 ng) was amplified using the AmpFISTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA) using the manufacturer's protocol. Each multiplex PCR reaction amplifies 16 short tandem repeat (STR) microsatellite loci from independent locations in the genome (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA). Each of the PCR primers is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, allowing the simultaneous resolution of 16 amplicons of similar size. PCR products were resolved by capillary gel electrophoresis and detected using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA). The height of each fluorescence peak in the electropherograms was quantitated using the ABI Prism GeneScan and Genotype Analysis software (Applied Biosystems, Foster City, CA) and a ratio of the peak heights of each pair of heterozygous allelic amplicons was calculated. By convention, the allele with the greater fluorescence intensity was designated the numerator. Thus, the ratio was always ≥ 1.0 , with 1.0 representing the theoretical ratio for normal alleles. We previously defined an operational threshold of AI (i.e. ≥ 2 sites of AI) that could differentiate between a variety of normal and cancerous tissues independent of storage conditions (i.e. fresh, frozen or paraffin-embedded, formalin-fixed) [39]. Of the 118 normal specimens, only 1 (0.8%) specimen demonstrated ≥ 2 sites of AI. In contrast, of the 239 tumor specimens, 161 (67.4%) demonstrated ≥ 2 sites of AI.

Statistical methods

The mean number of sites of AI and TC distributions for histologically normal, ADH, DCIS and invasive carcinoma specimens were analyzed by non-parametric Rank Sums tests. Chi-square tests were used to determine differences for individual allelic frequencies between the DCIS and invasive groups. JMP[®] statistical package (SAS Institute, Cary, NC) was used for all analyses and *P*-values < 0.05 were considered to be significant.

Results

TC in histologically normal tissue and ADH lesions

TC was determined in 54 histologically normal breast tissues obtained from women who underwent reduction mammoplasty. TC was tightly regulated within these histologically normal breast tissues; 95% of these normal specimens fell within the range of 75–154% (Fig. 1), nearly identical to the 75–143% range previously reported in a diverse set of 70 specimens of normal tissue from multiple organ sites, including breast [22]. Next, TC was determined in a set of 10 ADH lesions. TC values in two specimens (20%) fell outside the 95% range found in the histologically normal specimens.

Telomere DNA content in a test cohort of breast tumors

TC next was determined in a cohort of 27 DCIS, 104 Stage I and 32 Stage IIA breast tumors. In contrast to the

histologically normal group, there was a wide range of TC distribution in the tumor specimens within the test cohort (Fig. 1). Of the 27 DCIS cases, 10 (37%) fell outside the normal range. Similarly, 44 of the 104 Stage I tumors (42%) and 14 of the 32 Stage IIA tumors (44%) fell outside the normal range. However, the DCIS specimens as a group had longer telomeres than the Stage I ($P = 0.0152$) and Stage IIA ($P = 0.0338$) tumors.

Telomere DNA content in a validation cohort of breast tumors

The results were validated in an independent population-based breast tumor cohort comprised of 494 specimens. TC was determined in 95 DCIS, 244 Stage I, 112 Stage IIA, 39 Stage IIB and 4 Stage IIIA breast tumors. Fifty-five of the 95 DCIS cases (58%), 127 of the 244 Stage I (52%), 65 of the 112 Stage IIA (58%), 20 of the 39 Stage IIB (51%) and 3 of the 4 Stage IIIA (75%) tumors fell outside of the

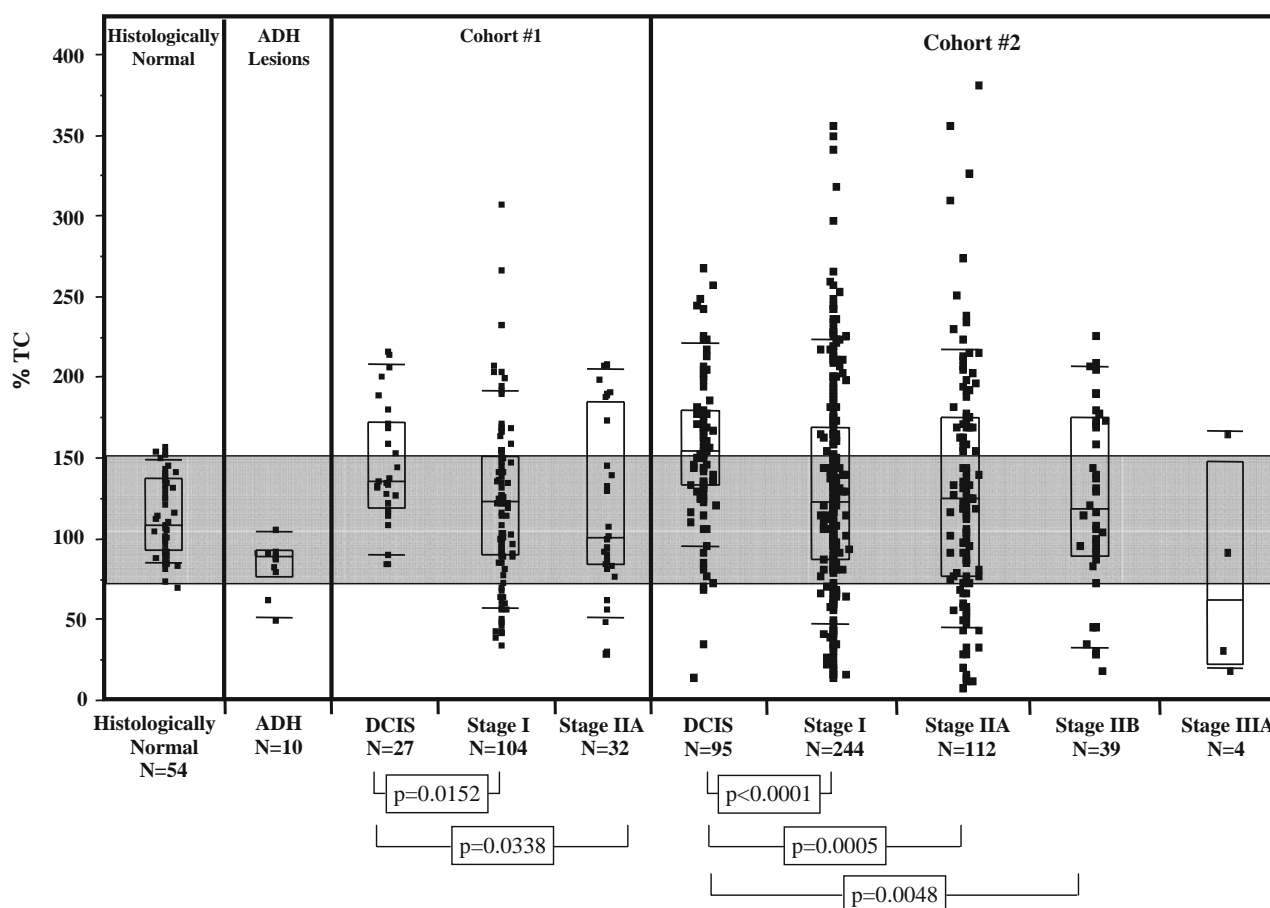


Fig. 1 TC distributions in histologically normal breast tissues derived from reduction mammoplasties, ADH lesions, and two independent cohorts of DCIS lesions and invasive breast carcinomas (Stage I–III). The numbers of tissues analyzed are indicated (N). TC is expressed as a ratio of TC in a placental DNA control. The boxes represent group medians (line across middle) and quartiles (25th and

75th percentiles) at its ends. Lines above and below boxes indicate 10th and 90th percentiles, respectively. The gray shaded area indicates 95% of TC measurements in the histologically normal group (75–154%). *Note:* Although the individual data points are horizontally shifted, some are still overlapping and therefore may not be visible

normal range defined by the histologically normal breast tissues. Again, the DCIS group had longer telomeres than the Stage I ($P < 0.0001$), Stage IIA ($P = 0.0005$) and Stage IIB ($P = 0.0048$) tumors (Fig. 1). In both the test and validation cohorts, TC did not correlate with ethnicity, nodal status, or ER and PR status.

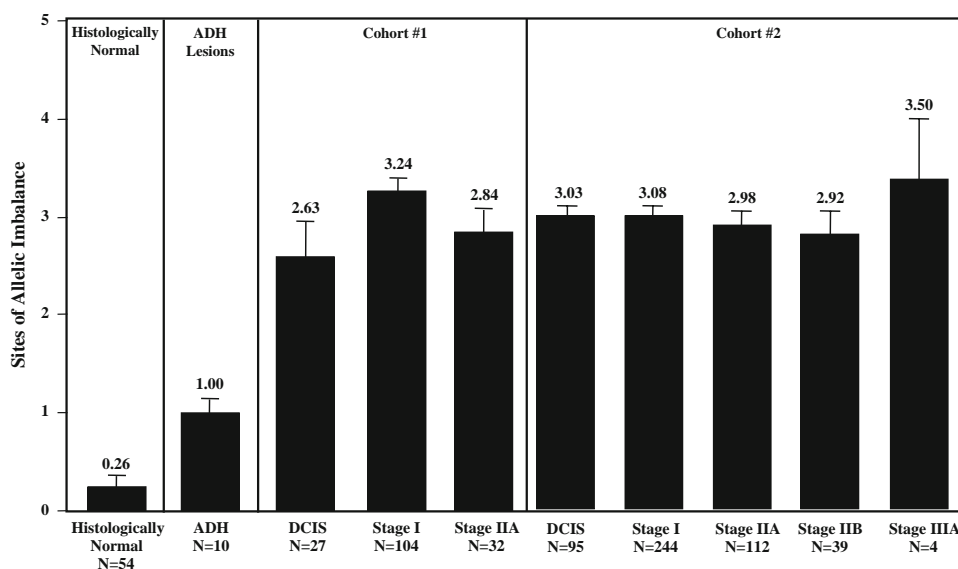
Extent of AI in histologically normal tissue and ADH lesions

To extend and confirm these findings, AI, another independent marker of genomic instability, was measured and compared in the same tissue cohorts. The mean number of sites of AI was 0.26 in the histologically normal and 1.00 in the ADH groups (Fig. 2). As compared to the histologically normal group, the ADH group showed a significant increase in the extent of AI ($P = 0.0002$), although the small number of ADH specimens must be noted.

Extent of AI in a test cohort of breast tumors

Next, the extent of AI was analyzed in the test cohort. The mean number of sites of AI was 2.63 in DCIS, 3.24 in Stage I tumors and 2.84 in Stage IIA tumors (Fig. 2). All groups were statistically different when compared to the histologically normal group ($P < 0.0001$ for each). As observed for TC, there was no difference in the extent of AI in the DCIS group compared to any of the invasive groups. Additionally, there was no difference between Stage I and Stage IIA tumors.

Fig. 2 Extent of allelic imbalance in histologically normal breast tissues derived from reduction mammoplasties, ADH lesions, and two independent cohorts of DCIS lesions and invasive breast carcinomas (Stage I–III). The numbers of tissues analyzed are indicated (N). The bars indicate the mean number of unbalanced loci (shown for each group) \pm standard errors. Abbreviations: ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ



Extent of AI in a validation cohort of breast tumors

These findings were replicated in the validation cohort. The mean number of sites of AI was 3.03 in DCIS, 3.08 in Stage I, 2.98 in Stage IIA, 2.92 in Stage IIB and 3.50 in Stage IIIA (Fig. 2). All categories were statistically different from the histologically normal group ($P < 0.001$ for each). There was no statistically significant difference between the DCIS group and the groups of invasive carcinoma or between any of the invasive groups. Additionally, there was no statistical difference in the mean number of sites of AI between paired groups by stage between the test and validation cohorts of breast tumors. Next, we tested our previously operationally-defined threshold for AI (i.e. ≥ 2 sites of AI) in these tissue cohorts [39]. Using this threshold, 0 of the 54 (0%) histologically normal breast specimens contained ≥ 2 sites of AI (Table 2). In contrast, 131 of the 163 tumors in the test cohort (80.4%) and 402 of the 494 tumors in validation cohort (81.4%) contained ≥ 2 sites of AI (Table 2). AI did not correlate with ethnicity, nodal status, or ER and PR status in both the test and validation cohorts.

Allelic frequency in DCIS and invasive tumors

Since the mean number of sites of AI in specimens of DCIS was nearly identical to the invasive tumors in both study cohorts, we next determined whether there was a difference in the allelic frequencies at each locus as a function of stage of progression. Since the individual loci have no known involvement in the development of breast cancer, there should be no selection pressure and the frequency of AI at a particular locus should not differ as a function of

Table 2 Extent of AI in cohorts of breast tissue

Group	<i>N</i>	# Samples ≥ 2 AI	%
Histologically normal	54	0	0.0
ADH	10	1	10.0
Cohort #1			
DCIS	27	20	74.1
Stage I	104	92	88.5
Stage IIA	32	19	59.4
Combined	163	131	80.4
Cohort #2			
DCIS	95	81	85.3
Stage I	244	196	80.3
Stage IIA	112	89	79.5
Stage IIB	39	32	82.1
Stage IIIA	4	4	100.0
Combined	494	402	81.4

Table 3 Frequency of AI at distinct loci in DCIS and invasive tumors

Loci designation	DCIS (<i>N</i> = 122)	Invasive (<i>N</i> = 535)	<i>P</i> value
D8S1179	0.19	0.26	0.108
D21S11	0.25	0.31	0.207
D7S820	0.03	0.08	0.066
CSF1PO	0.04	0.06	0.377
D3S1358	0.30	0.21	0.052
TH01	0.39	0.28	0.014
D13S317	0.18	0.24	0.175
D16S539	0.17	0.24	0.086
D2S1338	0.17	0.13	0.284
D19S433	0.14	0.18	0.310
vWA	0.35	0.32	0.538
TPOX	0.25	0.23	0.572
D18S51	0.10	0.10	1.000
D5S818	0.22	0.27	0.277
FGA	0.12	0.15	0.512

progression. For this analysis, the DCIS and invasive tumors were combined from the two tumor cohorts. As shown in Table 3, there were no statistically significant differences in the allelic frequencies of 14 of the 15 markers between the DCIS and invasive groups, except at the TH01 locus which showed an increase in AI in the DCIS samples compared to the invasive tumors ($P = 0.014$).

Discussion

The accumulation of genomic instability is characteristic of all carcinomas, including breast [1]. It has been proposed

that breast cancer progression can be modeled as a sequence of events progressing from normal epithelium to ADH, to DCIS, to finally the development of an invasive breast carcinoma [2, 3]. However, the genetic changes that underpin these histological changes still remain to be fully understood.

In this investigation we used TC and AI, two independent quantitative markers of genomic instability, to demonstrate that genomic instability increases as a function of the extent of breast disease (i.e. histologically normal tissue to ADH to DCIS). Alterations in TC and the extent of AI plateau in DCIS and do not increase further with increasing stage in invasive carcinomas. However, TC measurements show further telomere shortening between DCIS lesions and invasive carcinomas. The later finding is consistent with our previous studies demonstrating low TC compared to high TC confers an adjusted relative hazard of 4.43 (95% CI 1.4–13.6, $P = 0.009$) [22] in a cohort of 77 women. Additionally, in a population-based study of 530 women, low TC conferred an adjusted relative hazard of 2.88 (95% CI = 1.16–7.15; $P = 0.022$) [23].

Our TC findings are consistent with our previous reports that TC correlates with Stage in invasive carcinomas [22]. Here, we show that 95% of the histologically normal breast tissues analyzed in this study fall within a range of 75–154% of the placental DNA control, nearly identical to the range previously reported [21], demonstrating that TC is tightly regulated regardless of inherent tissue properties that may affect TC, such as organ site or patients' age. However, evidence of telomere dysregulation (i.e. attrition or elongation) was present in all the tumor cohorts. Speculatively, the finding of telomere elongation in tumors reflects the reactivation of telomerase, which is reactivated in 85–90% of tumors [40]. However, the extent of reactivation varies amongst tumors as demonstrated by Hines and colleagues who showed an approximate 800-fold difference in telomerase expression among a panel of 36 breast tumors [41]. Additionally, it has been postulated that early telomerase activation results in longer telomeres as compared to late activation, thus providing an opportunity for continued telomere shortening and accumulation of genomic instability.

Our observations confirm and extend the results of Ellsworth et al. [35] which demonstrated that levels of genomic instability are equivalent in DCIS lesions and advanced invasive tumors. However, that particular study utilized a panel of markers that were previously identified as important genes in the development of breast cancer. This confounds the ability to clearly interpret AI across these markers as genomic instability since these markers may be linked to oncogenes or tumor suppressor genes involved in the development of breast cancer. In contrast, the assay used in this study is based on AI at 16 random

microsatellite regions that have no known involvement in the development of breast cancer, and thus reflect genomic instability independent of their linkage to genes involved with breast tumorigenesis. The differences in the extent of imbalance among the particular loci may reflect the proximity of the microsatellite region to the telomere ends. Chromosomal differences in telomere length may also contribute to the individual heterogeneity.

In conclusion, the level of genomic instability assessed by (i) dysregulation in TC (i.e. outside the 95% range found in normal breast tissue) and (ii) extent of AI assessed at 16 microsatellite loci located throughout the genome, increases along the continuum of breast disease from histologically normal, to ADH lesions to DCIS and the level of genomic instability did not differ between DCIS and invasive carcinomas. In all, these findings suggest that DCIS lesions have the same extent of genomic instability (i.e. TC alterations and increased AI) as invasive carcinomas; thus supporting the notion that invasive carcinomas evolve from or in parallel with DCIS.

Acknowledgements This work was supported by grants DAMD17-01-1-0572, W81XWH-05-1-0226, W81XWH-05-1-0273 from the DOD Breast Cancer Research Program, NO-1-CN-65034-29 and SEER, NCI-PC-05016-20 from NCI/SEER and RR0164880 from the NIH. We thank Terry Mulcahy and Phillip Enriquez III from DNA Research Services of the University of New Mexico Health Sciences Center for gel capillary analysis.

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Table 1. Genes over-expressed in TAHN-1 compared to TAHN-5 tissues.

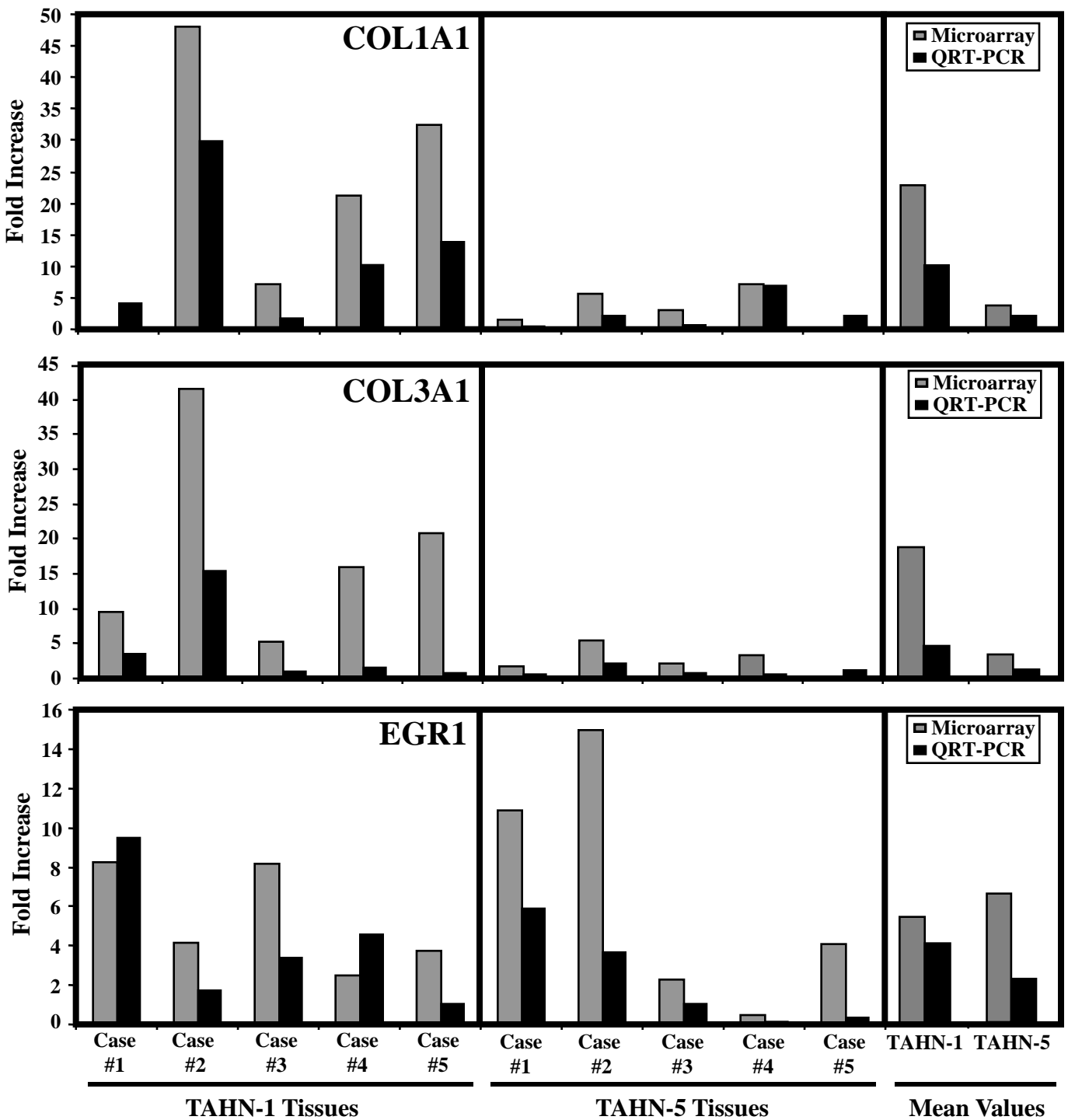
Transcript	1cm	5cm	1cm/5cm Ratio	Related to BrCa
Collagen Alpha 1(I) Chain Precursor	26.81	4.33	6.19	(19, 20)
Collagen Alpha 1(III) Chain Precursor	16.00	2.73	5.87	(19, 21)
Collagen Alpha 2(I) Chain Precursor	13.56	2.26	5.99	(20)
Protein Tyrosine Phosphatase, Non-receptor Type	10.31	1.93	5.34	No
URB	6.77	.90	7.55	No
Proton Myo-inositol Co-transporter (HMIT)	5.76	2.40	2.40	No
Alpha-1 Protease Inhibitor	5.07	1.40	3.62	No
Sorting Nexin 22	4.89	2.10	2.33	No
Collagen Alpha 2(VI) Chain Precursor	4.40	1.19	3.70	(19, 22)
Osteonectin (ON)	4.22	1.79	2.36	(23, 24)
THY-1 Membrane Glycoprotein Precursor	3.97	1.40	2.83	No
Collagen Alpha 1(VI) Chain Precursor	3.93	0.83	4.76	(21, 22)
Putative Insulin-Like Growth Factor II Associated Protein	3.54	0.93	3.82	No
Type 1 Procollagen C-Proteinase Enhancer Protein	3.33	1.27	2.63	No
Synaptic Vesicle Membrane Protein VAT-1 Homolog	2.85	1.01	2.76	No
Collagen Alpha 3(VI) Chain Precursor	2.80	1.19	2.36	(21, 22)
Mesoderm Specific Transcript Homolog	2.74	0.68	4.03	No
AE-Binding Protein 1	2.62	1.00	2.62	No
Follistatin-related Protein 1 Precursor	2.57	0.90	2.87	No
Fibulin-1 Precursor	2.53	0.82	3.07	(25)
Matrix Metalloproteinase 2 (MMP-2)	2.48	0.64	3.90	(26, 27)
Von Willebrand Factor Precursor (VWF)	2.45	0.54	4.52	(28)
Vascular Endothelial Cell Growth Factor 165 Receptor	2.42	0.60	4.04	(29)
Microfibril-Associated Glycoprotein 4 Precursor	2.27	0.94	2.41	No
Insulin-Like Growth Factor IB Precursor (IGF-IB)	2.24	0.86	2.62	(30)
Laminin Beta-1 Chain Precursor	2.18	0.91	2.41	(31)
Laminin Alpha-4 Chain Precursor	2.15	0.91	2.36	(31)
Tenascin XB	2.11	0.85	2.48	(32)
Fibronectin	3.23	1.46	2.21	(32, 33)
Collagen Alpha 1(XII) Chain Precursor	2.06	0.86	2.38	No
Phosphatidic Acid Phosphatase Type 2A	2.04	0.72	2.85	No

Table 2. Genes under-expressed in TAHN-1 compared to TAHN-5 tissues.

Transcript	1cm	5cm	1cm/5cm Ratio	Related to BrCa
Leptin Precursor	0.32	0.67	0.48	(34)
Fatty Acid-binding Protein, Epidermal (E-FABP)	0.46	0.88	0.53	(35)
Protein Kinase C Inhibitor Protein-1 (KCIP-1)	0.88	1.66	0.53	No
40S Ribosomal Protein S14	0.91	2.17	0.42	No
Decarboxylating, 6-Phosphogluconate Dehydrogenase	0.91	1.80	0.51	No

Table 3. Genes 4-fold over-expressed in TAHN-1 and TAHN-5 tissues compared to normal breast tissue.

Transcript	1cm	5cm	Related to BrCa
Collagen Alpha 1(I) Chain Precursor	26.81	4.33	(19, 20)
G25K GTP-Binding Protein (CDC42 Homolog)	7.05	4.36	No
Epsin 2 Isoform B; EPS15 Binding Protein	7.00	4.27	No
Scan Domain-Containing Protein 2 Isoform 1	6.91	4.61	No
OK/SW-CL.87	6.82	5.41	No
Connective Tissue Growth Factor Precursor	6.56	5.91	(36)
RAB-Like Protein 2A	6.28	4.63	No
Fuse Binding Protein 3	6.26	4.33	No
Cytochrome P450 3A43	5.78	4.51	No
Small EDRK-Rich Factor 1A	5.50	4.58	No
Ataxin-7	5.30	4.03	No
Focal Adhesion Kinase 1 (FADK1)	5.27	4.49	(37, 38)
Mitochondrial Ribosomal Protein L14	4.84	4.17	No
FUT1	4.73	4.48	No
Zinc Finger Protein 36	4.67	5.43	No
CYR61 Insulin-like Growth Factor Binding Protein	4.63	4.11	(39, 40)
Putative P150	4.50	4.35	No
Hereditary Hemochromatosis Protein Precursor (HLA-H)	4.28	4.52	No
Cisplatin Resistance-Associate Overexpressed Protein	4.25	4.94	No
Early Growth Response Protein 1 (EGR1)	4.17	4.08	(41, 42)
Lamin-Like Protein	4.12	4.19	No
Cytokeratin 19	4.08	4.41	(43)
Lipoate-Protein Ligase	4.01	5.25	No



Validation of microarray data by qRT-PCR. The results for COL1A1 (upper panel), COL3A1 (middle panel) and EGR1 (lower panel) are shown. TAHN-1 tissues are shown on the left, TAHN-5 tissues are shown in the middle, and the mean values for the two groups are shown on the right. The x-axis represents the fold increase of the transcript compared to the normal control RNA by microarray (gray bars) or qRT-PCR (black bars).

Telomere DNA Content Predicts Overall and Breast Cancer-free Survival Intervals

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BACKGROUND: There is a pressing need for new markers that accurately predict the likelihood of breast cancer recurrence. Telomeres are nucleoprotein complexes that protect chromosome ends from degradation and recombination. Critically shortened telomeres generate genomic instability. It has been postulated that the extent of telomere DNA loss is related to the degree of genomic instability within a tumor, and therefore may presage clinical outcome. The objective of this investigation was to evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissues predicts overall and breast cancer-free survival intervals.

METHODS: Slot blot titration assay was used to quantitate TC in archival breast tumor tissues from 530 members of the New Mexico subset of the NCI/SEER Health, Eating, Activity and Lifestyle (HEAL) prospective, population-based cohort. The relationships between TC and twelve risk factors for breast cancer adverse events (death due to breast cancer, breast cancer recurrence, or new primary breast tumor) were evaluated by Fisher's Exact Test. The relationships between TC, overall survival interval, and breast cancer-free survival interval were evaluated by log-rank analyses and displayed by Kaplan-Meier survival plots. Multivariate Cox proportional hazards models were used to evaluate the relationships between TC and twelve risk factors for breast cancer-free survival interval.

RESULTS: TC was independent of each of the twelve risk factors. Ethnicity, TNM stage, ER, PR and p53 status, chemotherapy sequence, adjuvant therapy, and TC each conferred significant relative hazards. The best overall multivariate Cox proportional hazards model included TC, p53 status, TNM stage, and ER status as independent predictors of breast cancer-free survival interval ($p < 0.00005$). Low TC ($\leq 200\%$ of standard), relative to the high TC group ($> 200\%$ of standard), conferred an adjusted relative hazard of 2.88 (95% CI=1.16-7.15; $p=0.022$) for breast cancer-related adverse events.

CONCLUSIONS: TC in breast cancer tissue is an independent predictor in this group of overall and breast cancer-free survival intervals.