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14. ABSTRACT

We have shown in retrospective studies that loss of telomere 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 is 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. 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. Currently, the candidate is assessing the potential prognostic capabilities of the assay in node negative breast tumors. 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 1cm 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, are on schedule.

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A – International Journal of Cancer accepted manuscript
B – Breast Cancer Research and Treatment accepted manuscript
C – Cancer Epidemiology, Biomarkers and Prevention submitted manuscript
D – Experimental Biology abstract
E – Era of Hope abstract
F – Prostate Cancer: Roadmap to the Future abstract
G – San Antonio Breast Cancer Symposium abstract

I. INTRODUCTION

Our laboratory has shown in retrospective studies that loss of telomere 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 PCR, is desirable. The aim of this study is 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 will provide 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, are on schedule.

Hypothesis and Rationale

Our preliminary results suggest that the extent of AI may have prognostic value in breast cancer. Consistent with this notion, Kronenwett and colleagues recently showed 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 hypothesize 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 will assess the potential prognostic value of AI in human breast tumor samples. Additionally, we propose to study AI in coexisting histologically normal (CHN) breast tissue and in stromal and epithelial cell populations. This hypothesis will be 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 first year of this training grant, I have demonstrated the following:

- Unbalanced allelic loci and altered telomere length 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 are conserved between the surrounding breast tissues and the tumors, implying cellular clonal evolution (Appendices A, E, G).

- Confirmed in a large cohort that TC in breast cancer tissue is an independent predictor of clinical outcome and may discriminate by stage (Appendix B).

- 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 (Appendix C).

- Validated findings in the breast by performing studies in the prostate, another hormone-dependent organ. The extent of AI in the normal disease-free tissues was substantially lower compared to the tumor and CHN tissues; however, AI and TC were similar in the tumor and CHN tissues. Additionally, reduced TC in prostate tissues obtained at radical prostatectomy predicts prostate cancer recurrence, independent of age at diagnosis, Gleason sum, and pelvic node involvement (Appendices D, F).

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the activation of this award, the Ph.D. candidate has been provided the opportunity to work and interact with oncologists, surgeons, pathologists and other Ph.D. scientists who all specialize in breast cancer. The candidate has attended journal clubs, specialized seminars and tumor board meetings. The candidate's research is overseen by his dissertation committee, a group comprised of three Ph.D. scientists with interests in breast cancer, and one M.D. that specializes in breast cancer pathology.

On an educational level, the candidate has helped instruct three upper-level Biochemistry courses: (1) Biochemical Methods Laboratory (2) Intensive Biochemistry I (3) Intensive Biochemistry II: Intermediary Metabolism. The candidate plans on continuing his breast cancer research in an academic setting, thus these 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 **In Progress**

- o 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.
- \- To date, 184 node negative breast tumors have been procured from NMTR. Collection of another 30 samples with disease recurrence is in progress.

Task 2 Months 12-14 **In Progress**

- o Isolate DNA from the paraffin-embedded breast tumors.
- \- DNA has been isolated from all 184 specimens.

Task 3 Months 14-24 **In Progress**

- o Measure AI in the paraffin-embedded breast tumors.
- \- AI has been successfully determined in 172 of the 184 samples.

Task 4 Months 24-30 **In Progress**

- o Analyze the correlation between the AI and patient recurrence status.

Specific Aim 2 (7 tasks)

Task 1 Months 1-30 **In Progress**

- o Prospectively, collect mastectomies and CHN breast tissues (1cm and 5cm from visible tumor margins).
- \- To date, 17 cases (tumor, 1cm, 5cm tissues) have been collected.

Task 2 Months 6-32 **In Progress**

- o 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.
- \- The pathological stage and grade have been assessed on 12 of the 17 cases.

Task 3 Months 6-32 **In Progress**

- o Isolate genomic DNA from tumor and CHN tissue specimens and determine extent of AI as a function of distance from tumor margin.
- \- Isolation of genomic DNA and determination of the extent of AI has been determined in 12 of the 17 cases (Appendices A, E, G)

Task 4 Months 6-32 **In Progress**

- o Isolate stromal and epithelial cell populations from selected CHN tissue specimens by LCM.
- \- The candidate has completed training for use of the LCM machine.

Task 5 Months 6-32 **Not Initiated**

- o Extract RNA from isolated cell populations.
- RNA will be extracted from the isolated cell populations this upcoming year.

Task 6 Months 9-32 **Not Initiated**

- o Measure extent of AI in epithelial and stromal cell populations.
- AI in these cell populations will be determined this upcoming year.

Task 7 Months 12-32 **Not Initiated**

- o 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.

Specific Aim 3 (5 tasks)

Task 1 Months 1-12 **In Progress**

- o 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.
- To date, 17 cases (tumor, 1cm, 5cm tissues) have been collected.

Task 2 Months 6-18 **In Progress**

- o 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.
- The pathological stage and grade have been assessed on 12 of the 17 cases.

Task 3 Months 6-18 **In Progress**

- o Isolate DNA from breast tumors and measure AI.
- Isolation of genomic DNA and determination of the extent of AI has been determined in 12 of the 17 cases (Appendices A, E, G)

Task 4 Months 24-30 **In Progress**

- o Analyze the correlation between the AI and pathological stage.

Task 5 Months 18-36 **In Progress**

- o Prepare and submit manuscripts.
- Two manuscripts have been accepted for publication and are currently in press (Appendices A, B) and one additional manuscript has been submitted for review (Appendix C).

Education and Training Milestones (6 tasks)

Task 1 Months 1-6 **In Progress**

- 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 **In Progress**

- 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 **In Progress**

- Interact with oncologists (Dr. Aroop Mangalik) in the University of New Mexico Hospital to gain perspective on breast cancer research.

Task 4 Months 1-36 **In Progress**

- 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.

Task 5 Months 12-18 **Not Initiated**

- Attend the University of New Mexico School of Medicine Undergraduate Medical Education Curriculum Neoplasia block.
- The candidate will attend the Neoplasia block with upcoming academic year.

Task 6 Months 12-36 **In Progress**

- Present ongoing work at local and national meetings.
- The candidate has presented work at three national meetings, two poster presentations (Appendices E, G) and an oral presentation (Appendix F), and has been a co-author on another poster presentation (Appendix D).

III. REPORTABLE OUTCOMES

Publications: (manuscripts in Appendix)
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, 2006, in press (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 DNA content in the staging and prognosis of breast cancer. Breast Cancer Research and Treatment, 2006, in press (Appendix B). **Authors contributed equally to this study*

Submitted Manuscripts: (manuscript in Appendix)
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. Cancer Epidemiology, Biomarkers and Prevention, 2006 (Appendix C).

Presentations: (abstracts in Appendix)
J.L. Wyaco, C.M. Heaphy, M. Bisoffi and J.K. Griffith (2005) Telomeric DNA Content and Allelic Imbalance in Normal, Tumor-Adjacent Histologically Normal and Tumor Prostate Tissue. Experimental Biology. San Diego, CA (Appendix D).

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 (Appendix E).

C.M. Heaphy, C.A. Fordyce, N.E. Joste, A.Y. Smith, W.C. Hunt and J.K. Griffith (2005) Association between cancer-free survival and telomere DNA content in prostate tumors. Prostate Cancer – Roadmap to the Future. Niagara Falls, NY (Appendix F).

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 (Appendix G).

IV. CONCLUSIONS

To date, all tasks; as outlined in the Statement of Work are on schedule. The tasks outlined in Specific Aim #1 have been initiated and are proceeding on schedule. To date, 184 node negative breast tumors have been obtained and analyzed, but we are still trying to procure specimens from patients with node negative breast cancer that have progressed to recurrent disease. The first 3 tasks of Specific Aim #2 have been initiated and a manuscript containing these results has been accepted for publication in the *International Journal of Cancer*, which is currently in press. The other 4 tasks of Specific Aim #2 have not yet been initiated, but work on these tasks will take place this coming year. The tasks outlined in Specific Aim #3 have been initiated and are proceeding on schedule. The Ph.D. candidate is progressing with all of his educational goals and is expecting to attend the neoplasia block at the University of New Mexico School of Medicine this upcoming semester.

APPENDIX A

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

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Key words: telomere loss, allelic imbalance, genomic instability, cancer field effect, breast cancer.
Category: Cancer genetics.

Novelty/Significance: Our study shows the occurrence of genetic alterations, such as telomere attrition and allelic imbalance, in histologically normal tissues adjacent to breast carcinomas, thus displaying field cancerization. Our study has practical implications for the assessment of surgical tumor margins and suggests that the evaluation of these margins should include molecular, in addition to histological, techniques.

Abstract

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 one centimeter 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.

Introduction

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 comprised 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 metastisis.[22] In agreement with this sequence of events, we and others have shown that telomere length, or its surrogate, telomere DNA content, 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 transformation of several types of cancer, including breast.[27,31,32] In addition, telomere attrition and other measures of genomic instability, such as allelic imbalance 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 allelic imbalance 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, telomere DNA content and allelic imbalance, 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 cm 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.

Material 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 summarized 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 cm and 5 cm from the visible tumor margins. After resection, the tissues were immediately frozen in liquid nitrogen. 10-12 μm sections 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 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.

Telomere DNA content (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 content of telomere DNA (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 1KB 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 previously described.[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, $(5'\text{-TTAGGG-}3')_4$-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 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.

Allelic imbalance (AI) assay

DNA (approximately 1 ng) was amplified using the AmpFlSTR 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.

Statistical analysis

Statistical analyses were performed using the JMP® statistical package (SAS Institute, Cary, NC) choosing a significance level of 0.01. The non-parametric 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.

Results

Telomeric DNA content (TC) in normal breast tissues

In order to define the normal range of TC in disease-free breast tissues, the content of telomere DNA (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 inter-quartile 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 peripheral blood lymphocytes (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) tissues are shown in Fig. 2. The first cohort was comprised of twelve 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. 2*A* and 2*D*). In contrast, both TA-HN-1 and TA-HN-5 tissues had normal architecture, lobular units, ducts, and adipose tissue (Fig. 2*B*, 2*C*, and 2*E*, 2*F*, respectively). Unlike the first cohort, which was comprised of snap frozen

tissues derived from contemporary mastectomies, the second was comprised 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. 2*G* and 2*I*) and TA-HN tissues (Fig. 2*H* and 2*J*). 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.

Telomeric DNA content (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 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 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 8/12 specimens of TA-HN-1 and 10/12 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), 16/38 TA-HN and 14/38 tumor specimens, respectively, had TC values less than those found in NBRST-RM tissues and PBLs, and only 9/38 TA-HN and 7/38 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 2), 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 micro-dissected, in a substantial fraction of the cells in the samples.

Allelic imbalance (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 allelic imbalance (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). Based on 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 Fig. 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, 3/20 and 1/12 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 two unbalanced loci, and none had more than two 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 two 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, indicate that at least one 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 micro-dissected, and the threshold for detecting AI requires that approximately 40% of the cells have lost the specific allele (see below), specific sites of AI are present in a substantial fraction of the cells.

Conservation of unbalanced alleles in tumor and adjacent breast tissues

In order 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 two cohorts of paired tumor and TA-HN tissues. As shown in Fig. 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 (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 $3x10^{-2}$ and 10^{-7} for the first and second cohorts, respectively.

Association between telomere DNA content and allelic imbalance in breast tumor tissues

Since telomere attrition is a source of genomic instability, and since we observed telomere attrition and increased allelic imbalance 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 two-sided Wilcoxon/Kruskal-Wallis Log Rank analysis revealed a significant difference in TC in tumors with high ($\geq$3 sites) as compared to low ($\leq$2 sites) allelic imbalance (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, telomere DNA content and allelic imbalance, 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 in 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 study in breast cancers that analyzes genomic instability at defined distances (1 cm 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 telomere DNA content and extent of allelic imbalance. Thus, we argue that telomere attrition induces genomic instability in breast tissues, and while this may not necessarily be apparent in histologically normal pre-cancerous 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. Based on DNA yields, we estimate that there were approximately 20 times more cells in the samples comprising the first cohort (median approximately 10^6 cells), than the second cohort (median approximately $5x10^4$ cells). This difference reflects the relative amounts of tissue available from the fresh surgical specimens comprising the first cohort versus the sections of paraffin-embedded tissue blocks comprising the second cohort. This consideration is particularly significant in the case of the AI assay. Based on theoretical considerations and mixing experiments (data not shown), we estimate that imbalance at a specific locus must occur in approximately 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, pre-malignant 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 due to 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 pre-cancerous 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 telomere DNA content.

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 pre-malignant 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 non-invasive 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.

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TABLE I - CLINICAL CHARACTERISTICS OF TUMOR COHORTS

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

[a] Dx, Diagnosis of invasive ductal carcinoma (IDC), lobular carcinoma (LC), ductal carcinoma in situ (DCIS); [b] S=small (≤2cm), L=large (>2cm); [c] N=negative, P=positive. TNM, Tumor-Nodes-Distant Metastasis; n/a, not applicable; n/av, not available.

TABLE II - TC VALUES IN NORMAL, TUMOR AND TUMOR ADJACENT, HISTOLOGICALLY NORMAL (TA-HN) TISSUES[a]

	N	Median	Mean	Min	Max	IQR[b]
Normal Tissues						
NBRST-RM[c]	20	126	127	114	158	12
PBL[d]	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

Notes: [a] Data from Figure 1; [b] IQR: Interquartile range; [c] NBRST-RM: Normal Breast tissue from Reduction Mammoplasty; [d] PBL: Peripheral Blood Lymphocytes.

Figure legends

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 cm 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 (25^{th} and 75^{th} percentiles) at its ends. Lines above and below boxes indicate 10^{th} and 90^{th} 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.

FIGURE 2 - Hematoxilin 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 μm.

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 cm 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 +/- 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.

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 (25^{th} and 75^{th} percentiles) at its ends. Lines above and below boxes indicate 10^{th} and 90^{th} percentiles, respectively. The non-parametric two-sided Wilcoxon/Kruskal-Wallis Log Rank test was used to assess the statistical significance of the difference between the means.

Figure 1

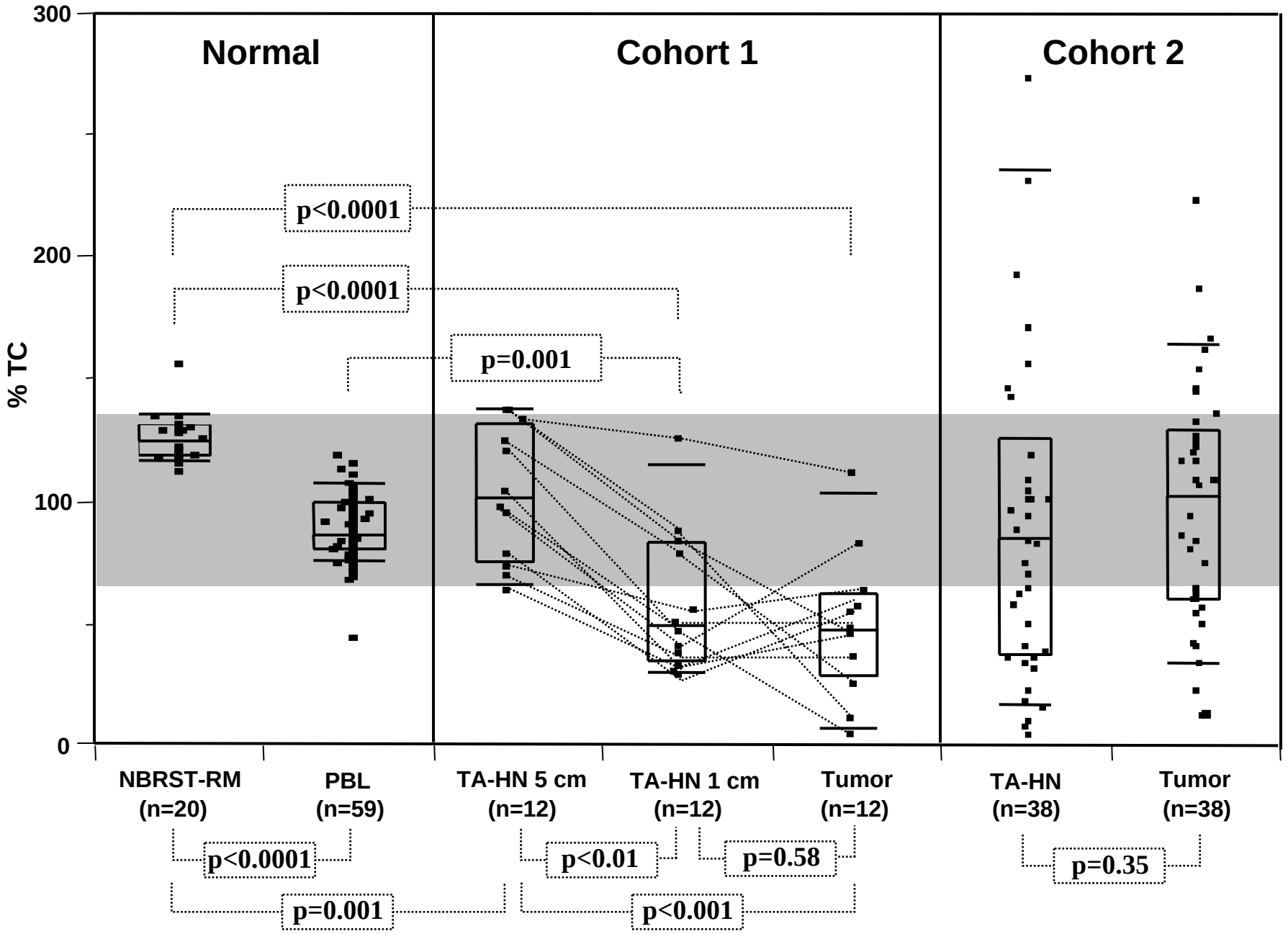
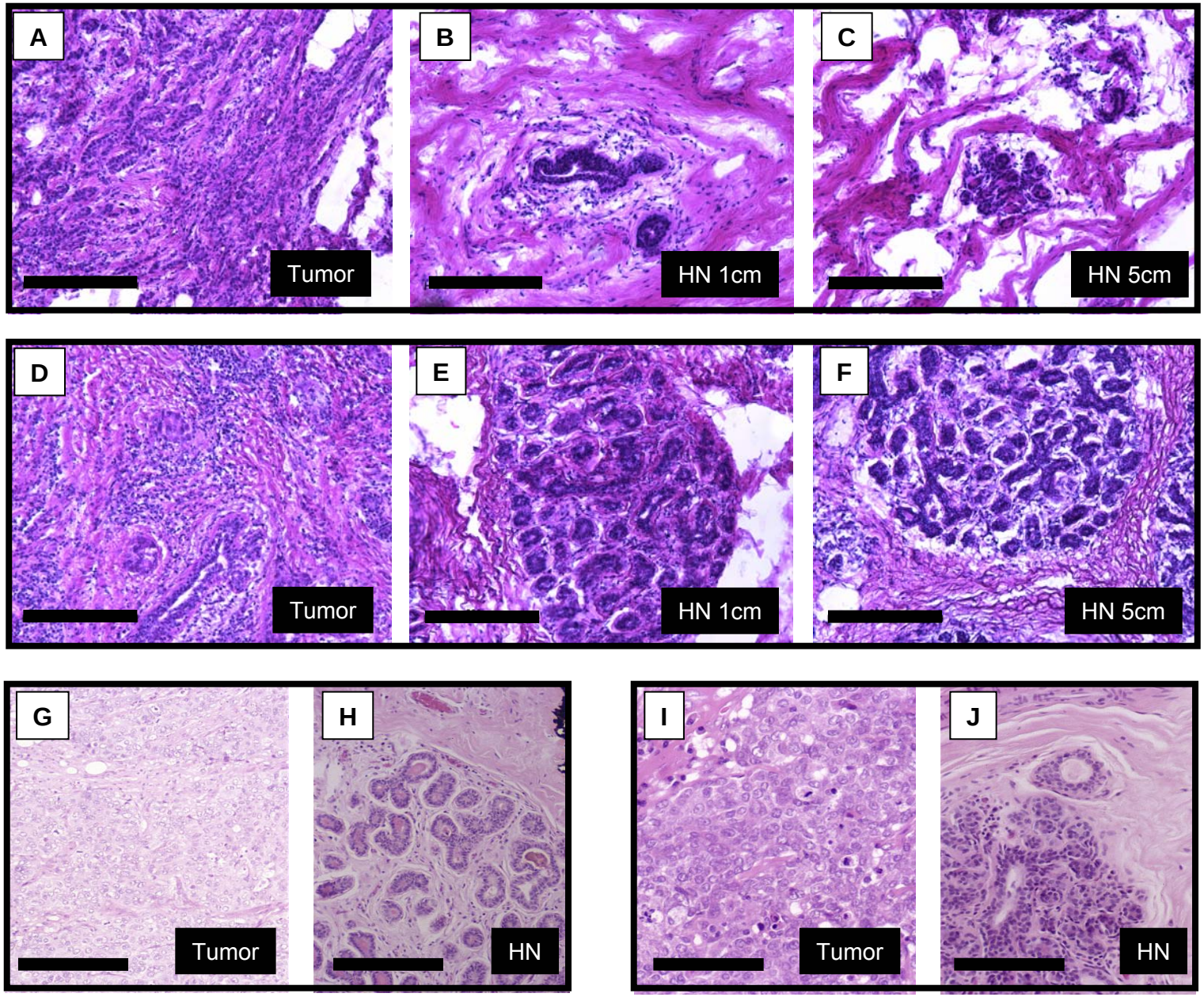

Normal
Cohort 1
Cohort 2
% TC
300
200
100
0
p<0.0001
p<0.0001
p=0.001
NBRST-RM (n=20)
PBL (n=59)
TA-HN 5 cm (n=12)
TA-HN 1 cm (n=12)
Tumor (n=12)
TA-HN (n=38)
Tumor (n=38)
p<0.0001
p=0.001
p<0.01
p=0.58
p<0.001
p=0.35

Figure 2


A
Tumor
B
HN 1cm
C
HN 5cm
D
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E
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F
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HN
I
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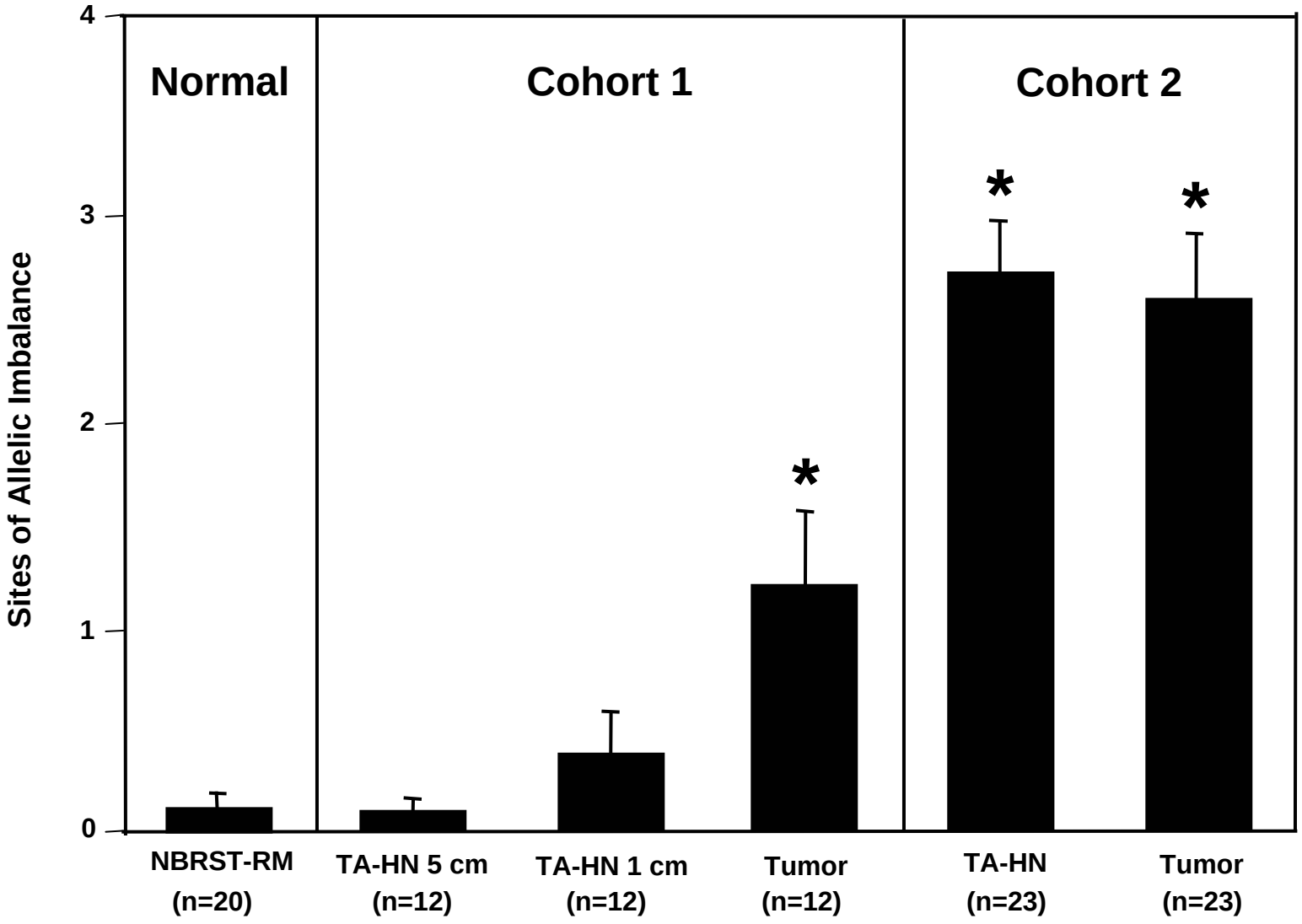
HN

Figure 3


Sites of Allelic Imbalance
4
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2
1
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Normal
Cohort 1
Cohort 2
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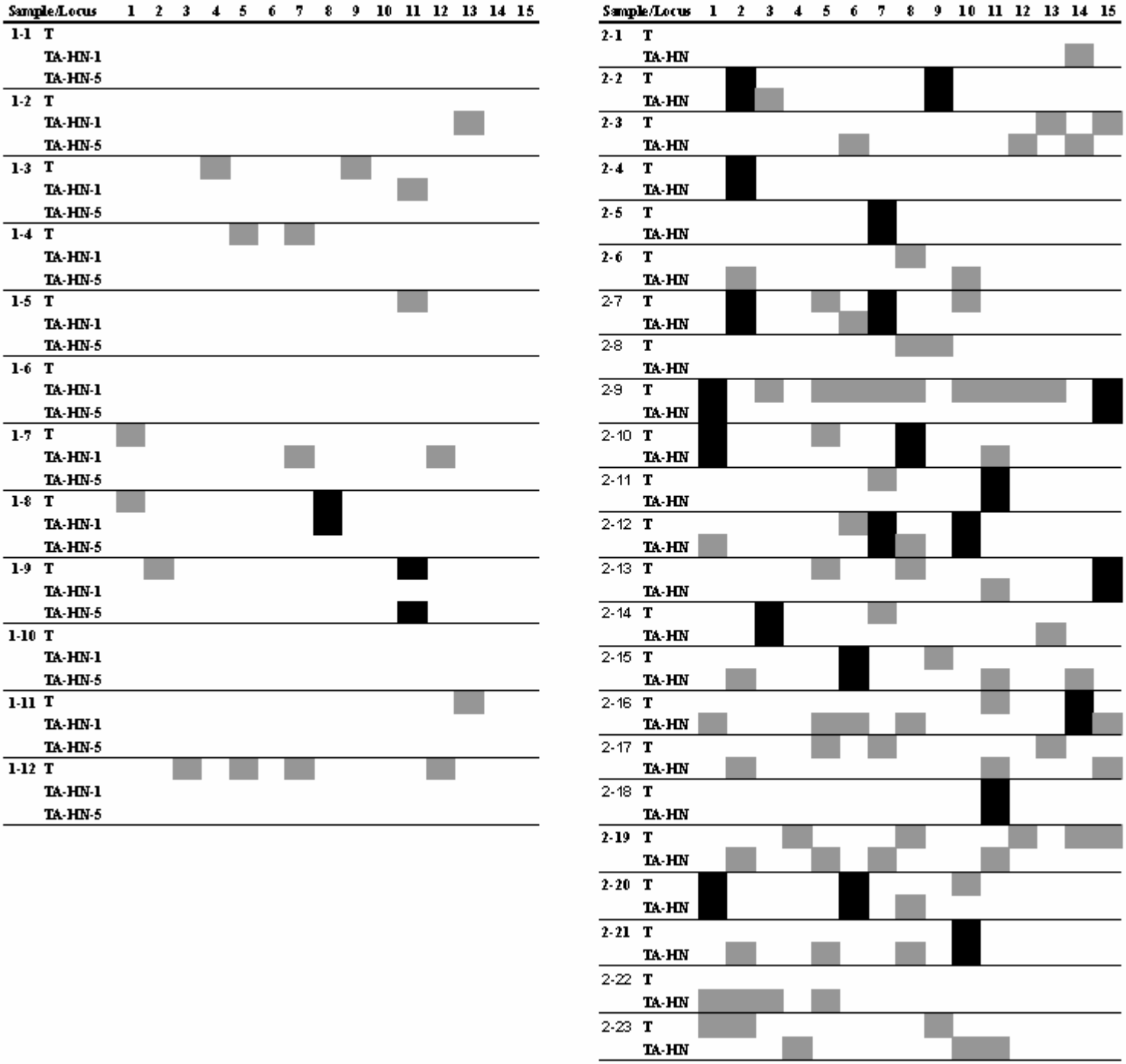
NBRST-RM
(n=20)
TA-HN 5 cm
(n=12)
TA-HN 1 cm
(n=12)
Tumor
(n=12)
TA-HN
(n=23)
Tumor
(n=23)

Figure 4


Sample/Locus 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
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TA-HN-1
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TA-HN-1
TA-HN-5
1-4 T
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Sample/Locus 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
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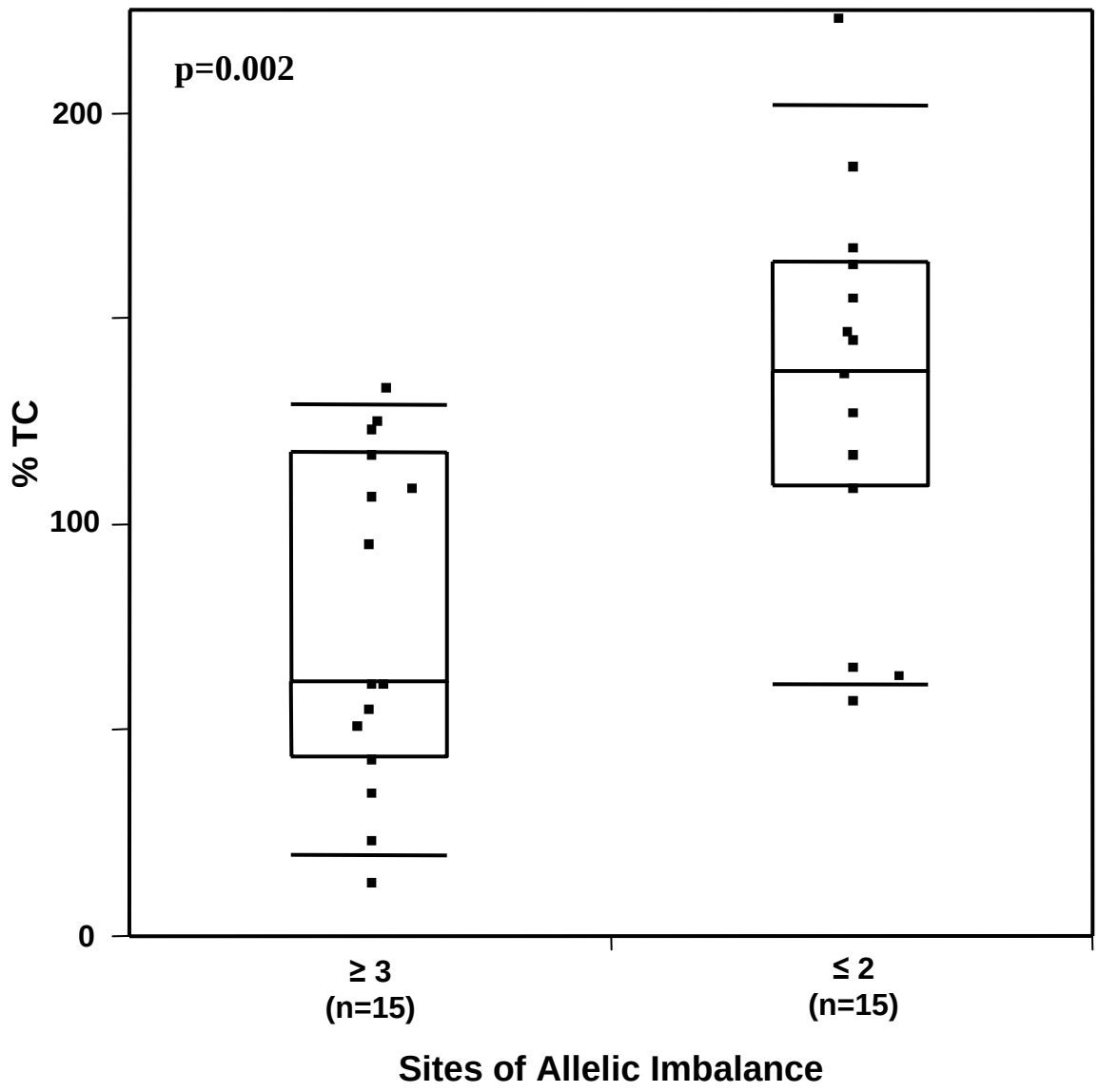
Figure 5



APPENDIX B

Telomere Content Correlates with Stage and Prognosis in Breast Cancer

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Running Title: Telomere Content in Breast Cancer Prognosis and Staging

Article Type: Original Laboratory Investigation

Keywords: Breast Cancer, Genomic Instability, Metastasis, Prognosis, Telomere, TNM Staging

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ABSTRACT

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 (**T**umor size-**N**odal involvement-**M**etastasis) 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 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), five-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 (’82-’93) 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 (’96-’99) 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 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, $(5'\text{-TTAGGG-}3')_4$-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 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 5cm from the breast tumor margins (HNB), buccal cells from healthy, young men and women (BUC) and peripheral blood lymphocytes 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 twenties, 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].

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 '82-'93 and '96-'99 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 '82-'93 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 '96-'99 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 '82-'93 and '96-'99 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 (Figures 2a-c) and logistic regressions (Figures 2f-h). In each instance, there were highly significant associations with TC. Approximately 85%

of the tumors in the ’82-‘93 set were TNM stage IIA or higher; while approximately 66% of tumors in the ’96-‘99 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 five years of follow-up data were available for 137 of the 140 women in the ’82-’93 and ’96-‘99 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 (Figures 2d, 2e) and logistic regressions (Figures 2i, 2j). 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 ’82-’93 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 ’96-’99 set was also associated with overall 5-year survival ($p=0.02$) however, no members of the ’96-’99 set died from breast cancer within five 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 ’82-’93 group, and overall 5-year survival in the ’96-’99 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 '82-'93 and '96-'99 sets, respectively, had TC values within the range defined by the middle tertile. The 124% cutoff predicted five 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 '82-'93 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; p=0.008) relative to high TC. A multivariable Cox model for the '82-'93 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 $p=0.0004$, respectively). TC thresholds based on the tertile distributions in normal tissues (described above) predicted five year breast cancer-free survival with approximately 50% sensitivity and 95% specificity and death resulting from breast cancer within five 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.

ACKNOWLEDMENTS

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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Table 1. Characteristics of tumor tissues.

Set[a]		Size (mm)					Node Involvement			TNM Stage								Number of Deaths at 5-years of follow up	
	N	Median	Mean	Range	Q1	Q3	Yes	No	NA	0	I	IIA	IIB	IIIA	IIIB	IV	NA	Total	Breast Cancer Related[b]
'82-'93	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%)
'96-'99	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%)

[a]Tissue sets are described in the Materials and Methods Section. [b] Of the 10 subjects who died from causes other than breast cancer, 6 died from other cancers, and 1 each from dementia, hypertension, pulmonary embolism, and unknown causes. Abbreviations: N: Number of specimens, Q1, Q3: First and 3rd quartile (the difference between Q1 and Q3 is the inter-quartile range, or IQR). NA: Not available.

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

Set[a]		Age at Tissue Collection					Telomere DNA Content (% Placental DNA Control)				
	N	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
'82-'93	77	48	52	31-88	42	60	108	109	36-247	77	126
'96-'99	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

[a]Tissue sets are described in the Materials and Methods Section. Additional details are found in the text and the legend to Figure 1. Abbreviations: N: Number of specimens, Q1, Q3: 1st and 3rd quartile (The difference between Q1 and Q3 is the interquartile range, or IQR). NA: Not available.

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

[a]Tissue sets are described in the Materials and Methods Section. 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.

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%	
	N	%	N	%	N	%
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
	N	Mean	N	Mean	N	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

[a]Size is measured in longest dimension. Abbreviations: TC: Telomere DNA content, N: Number of specimens.

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)	***p*-value**	**RH (95% CI)**	***p*-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.

FIGURE LEGENDS

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.

Figure 2. Associations Between Breast Tumors' Telomere DNA Contents (TC) and Tumor Size, Nodal Status, TNM Stage and Five Year Breast Cancer-free Survival. Tumor sets '82-'93 and '96-'99 were combined and stratified by tumor size (panel a), nodal status (panel b), TNM stage (panel c), overall 5-year survival (panel d) and breast cancer-free 5-year survival (panel 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 (panel f), nodal status (panel g), TNM stage (panel h) overall 5-year survival (panel i) and breast cancer-free 5-year survival (panel 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.

Figure 3. Receiver Operating Characteristics (ROC) Analysis of the Relationship Between TC in Breast Tumors and Five Year Survival. The specificity and sensitivity of TC as a predictor of five year survival following diagnosis of breast cancer was calculated for each value of TC in the combined'82-'93 and '96-'99 sets. Seventeen subjects died from breast cancer, and 119 survived for at least five 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 '82-'93 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.

Figure 1

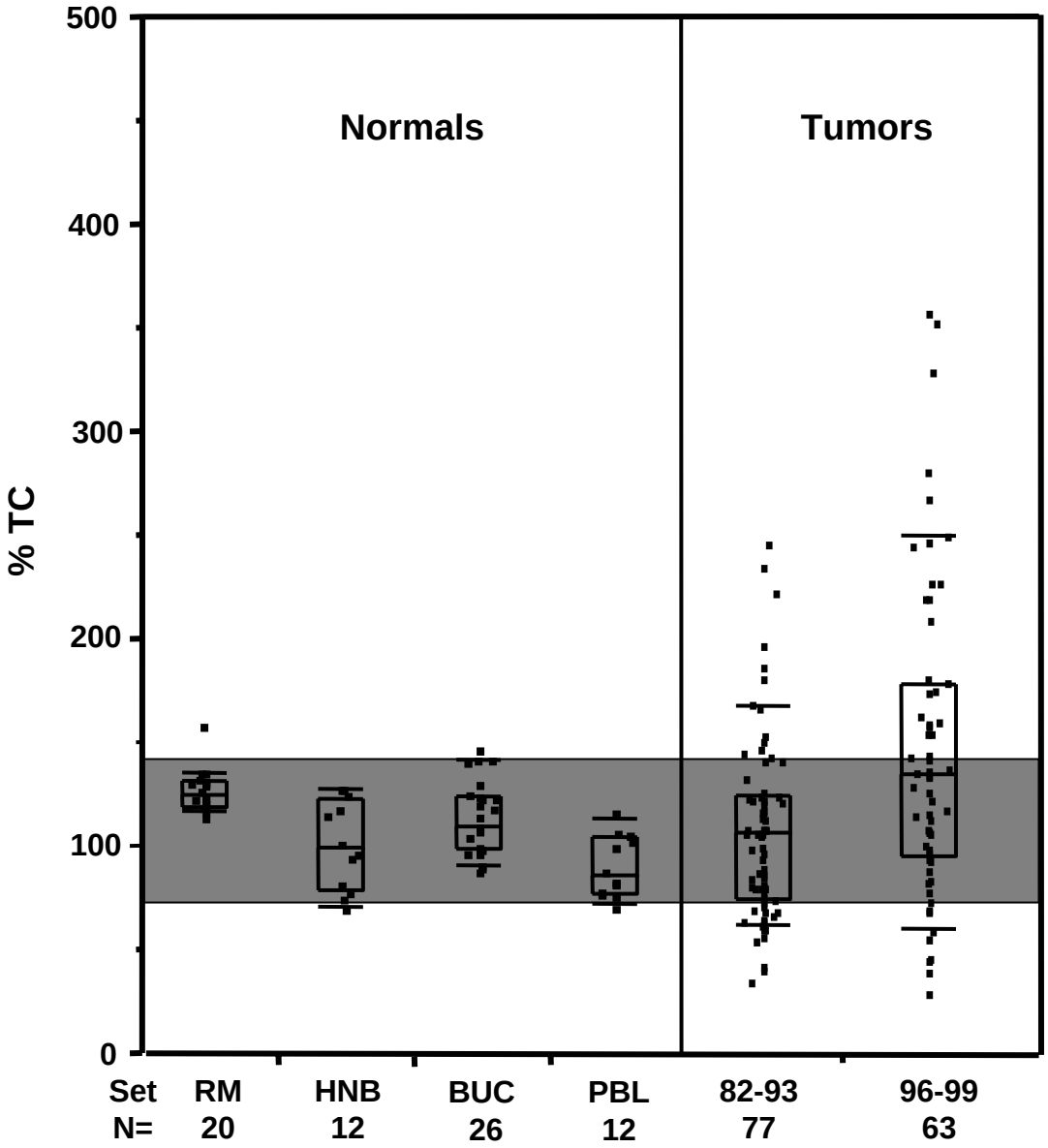

Normals
Tumors
% TC
500
400
300
200
100
0
Set
N=
RM
20
HNB
12
BUC
26
PBL
12
82-93
77
96-99
63

Figure 2

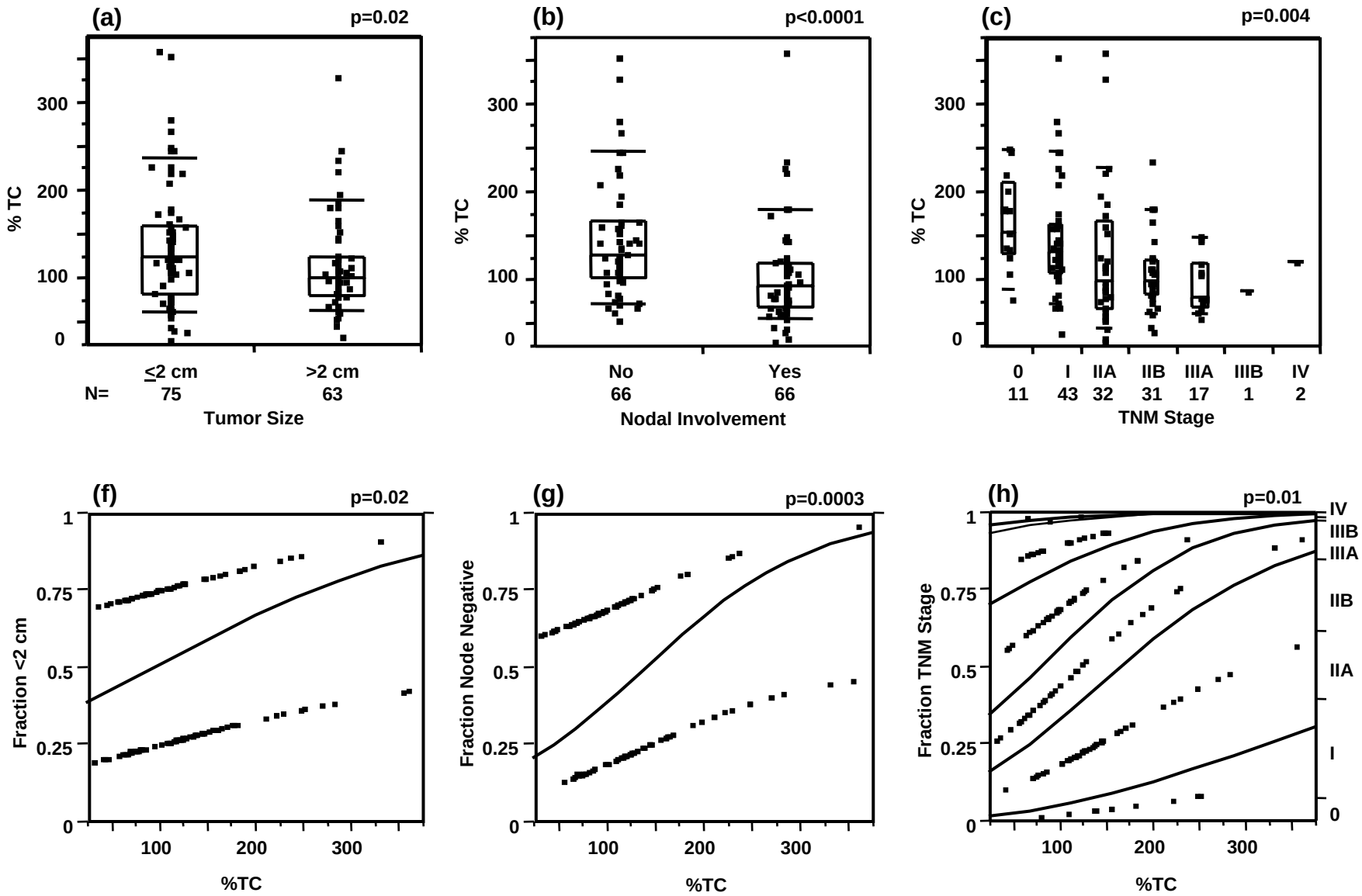
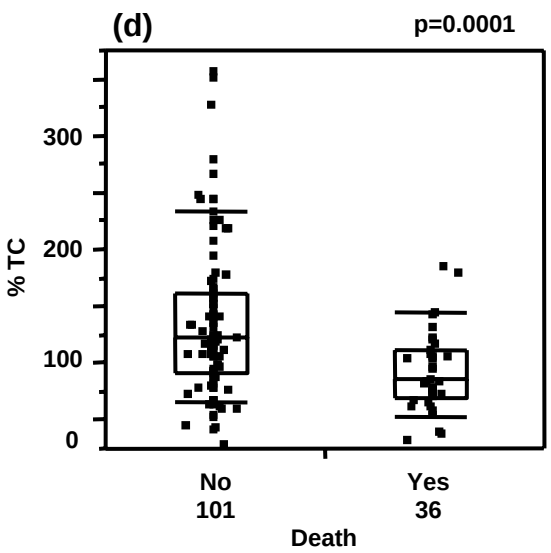
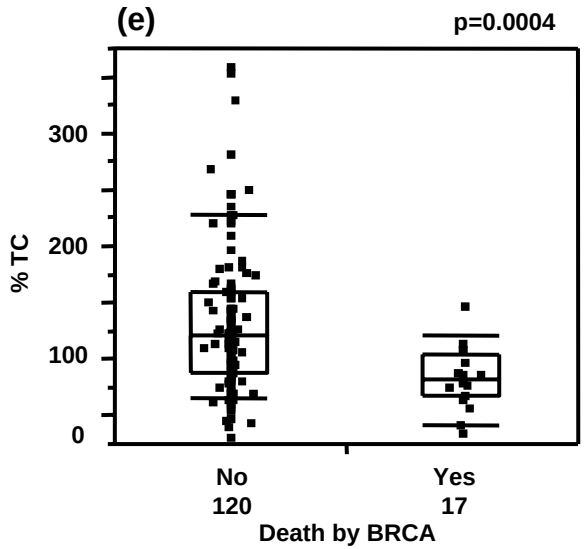
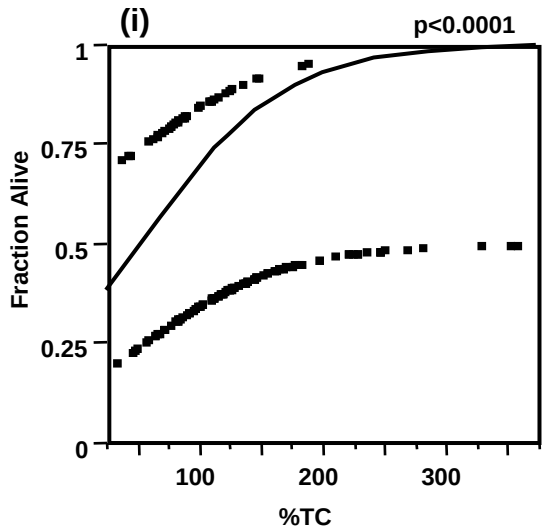

(a)
p=0.02
% TC
300
200
100
0
≤2 cm
>2 cm
N=
75
63
Tumor Size
(b)
p<0.0001
% TC
300
200
100
0
No
Yes
66
66
Nodal Involvement
(c)
p=0.004
% TC
300
200
100
0
0
I
IIA
IIB
IIIA
IIIB
IV
11
43
32
31
17
1
2
TNM Stage
(f)
p=0.02
Fraction <2 cm
1
0.75
0.5
0.25
0
100
200
300
%TC
(g)
p=0.0003
Fraction Node Negative
1
0.75
0.5
0.25
0
100
200
300
%TC
(h)
p=0.01
Fraction TNM Stage
1
0.75
0.5
0.25
0
IV
IIIB
IIIA
IIB
IIA
I
0
100
200
300
%TC

Figure 2 (cont.)







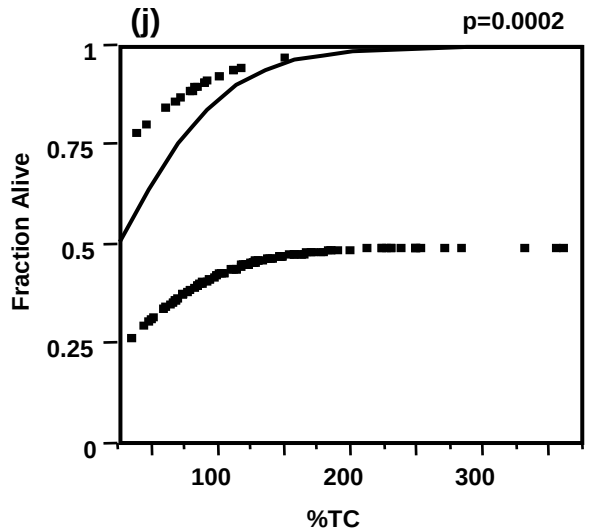


Figure 3

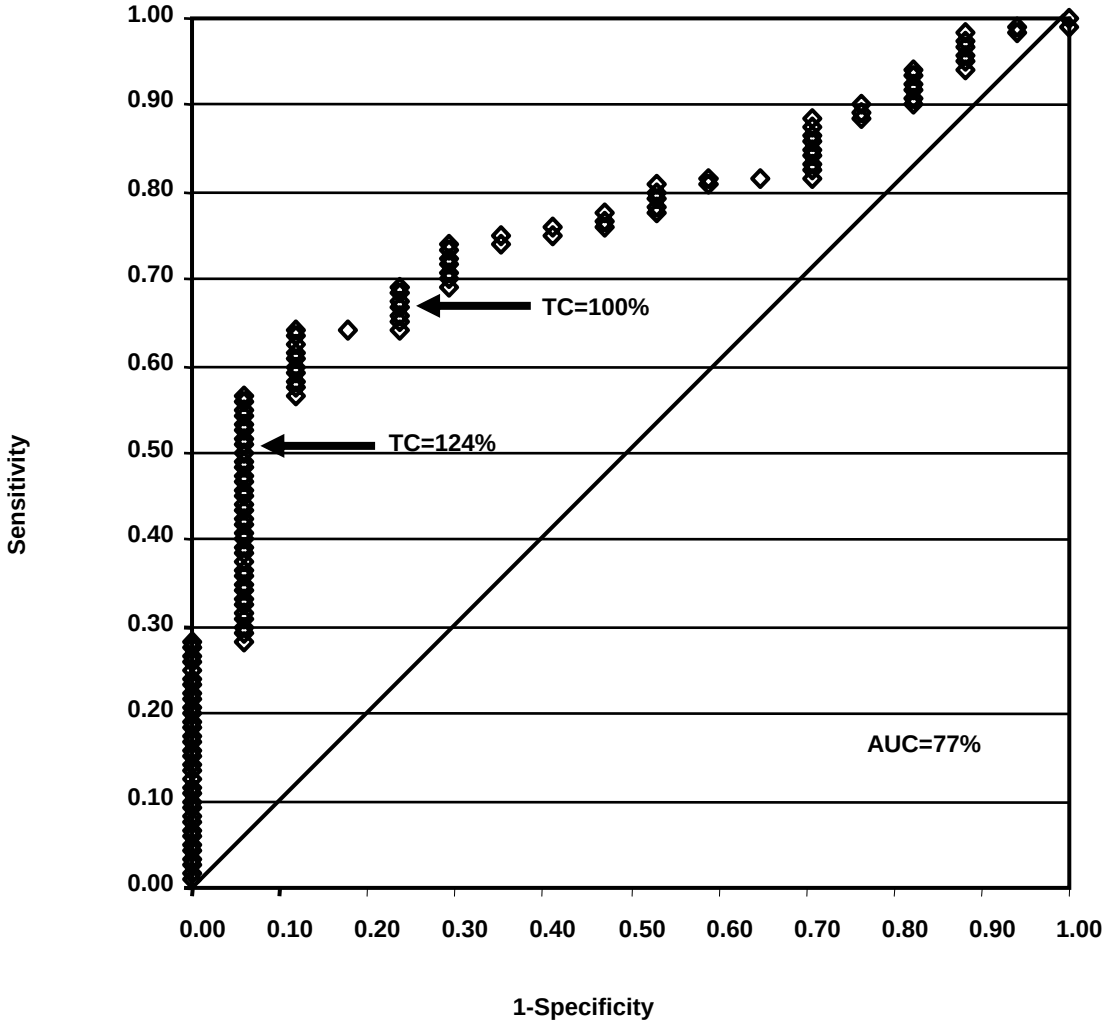
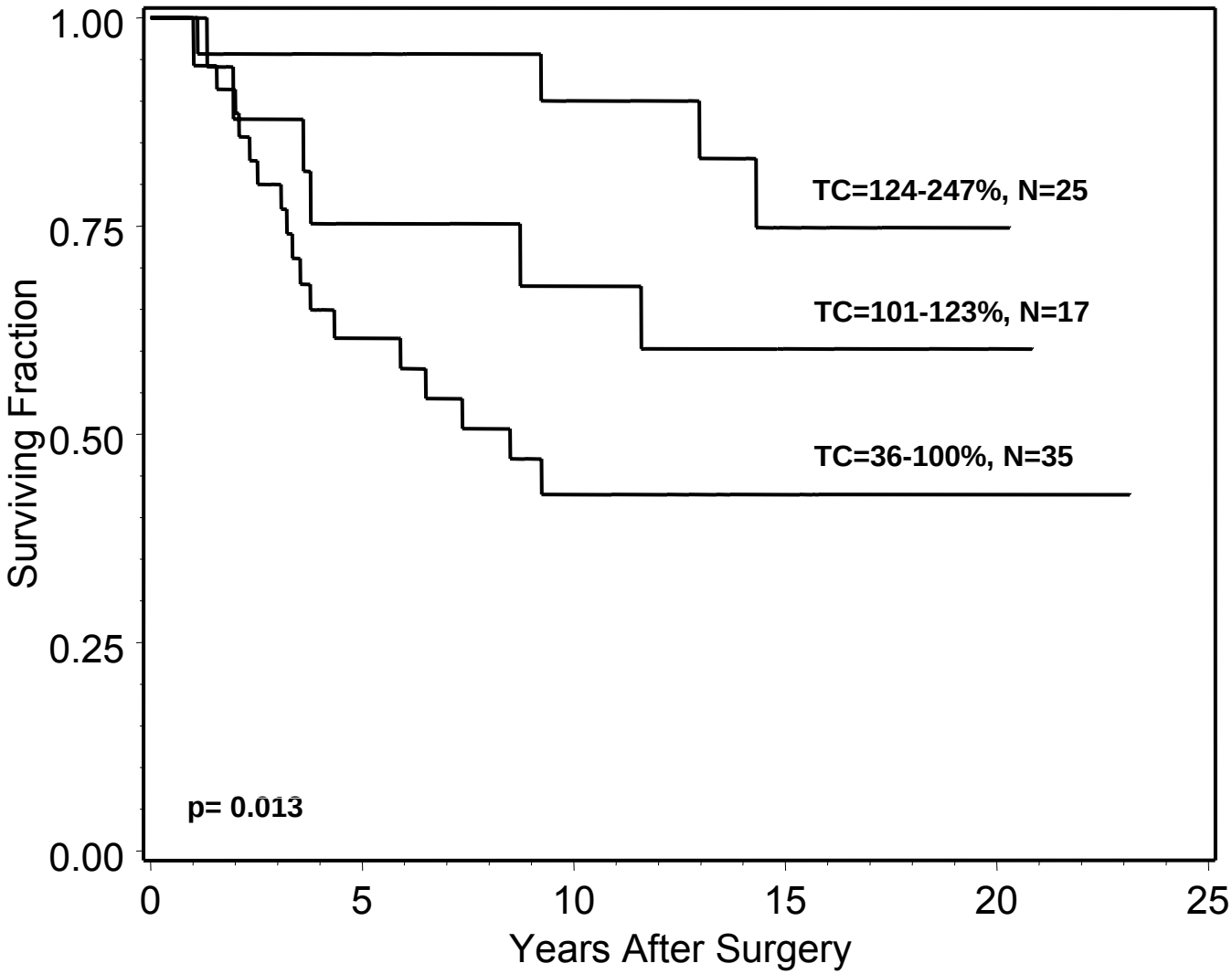

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Sensitivity
TC=100%
TC=124%
AUC=77%
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
1-Specificity

Figure 4


1.00
0.75
0.50
0.25
0.00
Surviving Fraction
0
5
10
15
20
25
Years After Surgery
TC=124-247%, N=25
TC=101-123%, N=17
TC=36-100%, N=35
p= 0.013

APPENDIX C

Measurement of Genome-wide Allelic Imbalance in Human Tissue Using a Multiplex PCR System

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Running Title: Measurement of Genome-wide AI in Human Tumors

Keywords: Allelic Imbalance, Genomic Instability, Human Tumors, Loss of Heterozygosity, Microsatellite DNA

Abstract

The most common causes of genomic instability generate chromosome breakage and fusion randomly throughout the genome, frequently resulting in allelic imbalance, which is any deviation from the normal 1:1 ratio of maternal and paternal alleles. It is reasonable to speculate that tumors with more sites of allelic imbalance have a greater likelihood of having disruption of any of the numerous critical genes that result in clinically-relevant phenotypes, such as invasion, extravasation and metastasis. For this reason, it is desirable to develop a robust method to assess and quantify the extent of allelic imbalance, thus providing a global assessment of genomic instability in any tumor. To address this need, we designed a high-throughput method, based on the Applied Biosystems AmpFℓSTR® Identifiler multiplex PCR 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 very little DNA (the equivalent of approximately 150 cells), and uses commercially available reagents, instrumentation and analysis software. Most importantly, this method is able to discriminate between normal and tumor specimens with 67% sensitivity and 99% specificity.

Introduction

It is widely accepted that genomic instability- the duplication, loss or structural rearrangement of a critical gene(s) - occurs in virtually all cancers (1), 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 that affect patterns of gene expression which regulate aspects of cell proliferation, apoptosis, angiogenesis, invasion and, ultimately, metastasis (2, 3).

There are numerous technologies available to detect allelic imbalance (AI), which is any deviation from the normal 1:1 ratio of maternal and paternal alleles, and loss of heterozygosity (LOH), which is the complete loss of either allele. For example, chromosome painting techniques can identify AI and LOH in cytological preparations (4, 5). However, 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 LOH detection (6-8). For example, the development of a panel of 52 microsatellite markers that detects genomic patterns of LOH (9-11) has been utilized for breast cancer diagnosis and prognosis. However, this approach requires referent (normal) DNA and these cancer-specific panels may not be informative for other cancer types, thus multiple panels are required.

Larger panels of SNPs may be used for genome-wide analysis, for example the Affymetrix 10K and 100K SNP mapping arrays (12, 13). Likewise, Illumina BeadArrays

with a SNP linkage-mapping panel (14), allow allelic discrimination directly on short genomic segments surrounding the SNPs of interest, thus overcoming the need for high-quality DNA (8). Lips and colleagues have shown that these Illumina BeadArrays can be used to obtain reliable genotyping and genome-wide LOH profiles from formalin-fixed paraffin-embedded (FFPE) normal and tumor tissue (15). However, both of these approaches, while robust, require costly reagents, specialized equipment, and the sheer amount of data produced from these analyses delay the interpretation of results.

For these reasons, it is desirable to develop a general, economical, and high-throughput method to provide a global assessment of genomic instability in any tumor, 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 PCR reaction. The assay, which is based on the Applied Biosystems AmpFℓSTR® Identifiler system, can be performed with only 1 ng of genomic DNA, and uses commercially available primers and reagents, and common instrumentation and analysis software. Thus, it is an attractive alternative that is readily adaptable to most clinical laboratory environments.

Materials and Methods

Tissue Acquisition: All normal and tumor 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 formalin-fixed, paraffin-embedded (FFPE) prostate tumors, obtained by radical prostatectomy, were provided by the Cooperative Prostate Cancer Tissue Resource (http://www.cpctr.cancer.gov). Duodenal and pancreatic FFPE 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 UNM 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 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.

Multiplex PCR Amplification of STR Loci: The AmpFℓSTR® Identifiler kit (Applied Biosystems, Foster City, CA) was used to amplify genomic DNA at 16 different short tandem repeat (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. 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, Foster City, CA). Fluorescent peak heights were quantified using ABI Prism GeneScan® Analysis software (Applied Biosystems, Foster City, CA). Allelic ratios were calculated using the peak height, rather than the peak area, as suggested in previous studies (16-18). 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 Chi-square test was performed using SAS JMP® software version 9.1 (SAS Institute Inc., Cary, NC) to examine the relationship between the extent of allelic imbalance and tissue type, using a significance level of 0.05.

Results

The 16 allelic microsatellite loci amplified by the AmpFℓSTR® Identifiler primer sets are unlinked, and can be used to assess allelic imbalance 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 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 loci, ostensibly due to different PCR efficiencies, the peak heights of the paired alleles were relatively identical. Theoretically, the ratio of any two heterozygous alleles following PCR amplification would be 1.0 in normal tissues. To test this premise, the ratios of paired alleles' signal intensities were compared at 320 heterozygous loci in buccal cells from 27 healthy individuals. As expected, the mean ratio was near 1.0 (mean =1.15, SD 0.18). We expect that approximately 97.5% of all allelic ratios would fall within 2.5 SD of the mean, and therefore operationally defined an allelic ratio >1.60 (mean + 2.5 SD) as a site of AI. Applying this threshold to the 27 analyzed buccal samples, only 8 sites of AI were detected out of the 320 heterozygous loci, thus representing a value of 0.30 sites of AI 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 >1.60.

In order to demonstrate that AI determinations were reproducible, allelic ratios were remeasured within a random subset of the buccal samples. Figure 2A shows that 193 of the 198 (97.5%) loci measured were correctly identified upon repeating the experiment; whereas, only 5 of the 198 (2.5%) loci initially designated as sites of AI could not be confirmed. 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.

In order to confirm that the differences in AI detected by this approach reflected true differences in the ratio of the alleles, and not experimental artifact, 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 (TH01: 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 a site of AI (data not shown).

To validate the operationally-defined threshold for AI, we measured the allelic ratios for 1382 heterozygous loci in an independent test set comprised of 118 normal samples consisting of bone (n=2), breast (n=10), buccal (n=53), lymph node (n=5), peripheral

blood lymphocytes (PBL) (n=18), pancreas (n=6), placenta (n=3), prostate (n=4), renal (n=16) and tonsil (n=1) tissues (Figure 3A). In summary, 88 (74.6%), 29 (24.6%), and 1 (0.8%) of the 118 normal tissues specimens contained 0, 1 and 2 loci with AI, respectively. In this sample set of normal tissues, only 32 of 1382 possible sites were designated sites of AI, thus representing a value of 0.27 sites of AI per sample, versus 0.30 sites of AI per sample in the original sample set.

We next compared this to the frequency of AI in 2792 heterozygous loci in an independent test set comprised of 239 frozen or paraffin-embedded tumor samples consisting of AML (n=8), breast (n=39), CML (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 0, 1 and $\geq$2 loci with AI, respectively. In contrast to the normal tissues, 611 sites of AI were detected, thus representing a mean of 2.56 unbalanced loci per sample, nearly 10 times greater than the frequency in the normal tissues ($p<0.0001$).

Discussion

Manifestations of genomic instability, such as AI and LOH, are widespread in solid tumors (1). There have been numerous studies of these abnormalities and several techniques, including chromosome painting and SNP arrays, have emerged to analyze these differences between normal and tumor tissues (4-13). However, these methods are typically costly or time intensive, or need a referent DNA (normal) sample for analysis. For this reason, it is desirable to develop a high-throughput method to quantify the extent of allelic imbalance in the genome of any tumor, independent of the nature and composition of the specimen and the availability of matched, normal tissue.

Using our PCR-based, high-throughput assay and newly developed interpretation scheme to score the extent of genome-wide unlinked allelic imbalance 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 to 0.8% of the normal samples, which represents an almost 84 fold difference. It must also be noted that this method provides a minimum estimate of AI, since the assay cannot discriminate between homozygous alleles and complete loss of heterozygousity in the absence of matched normal tissue. However, this limitation is mitigated by the near ubiquitous presence of normal tissue within tumors. For example, an allelic ratio of 1.60, our definition for a site of AI, could represent either a population comprised of 60% normal cells heterozygous for the allele and 40% tumor cells with complete loss of one allele, or 40% normal cells heterozygous for the allele and 60% tumor cells with duplication of one allele. The latter 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.

In conclusion, we describe here a simple method for measuring the extent of allelic imbalance throughout the genome. This method has a number of significant advantages over existing technologies, such as chromosome painting and SNP arrays. The advantages of this method are that: (i) it is robust, reproducible and provides a quantitative basis for comparing the extent of AI between samples; (ii) it does not require matched normal tissue; (iii) it utilizes commercially available reagents, instrumentation and analysis software; (iv) it can be applied to a variety of fresh, frozen and archival tissues; (v) it requires very little DNA (the equivalent of approximately 150 cells); and (vi) it discriminates between normal and tumor specimens with 67% sensitivity and 99% specificity.

Acknowledgements

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Figure Legends

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. Fluorescent intensity is shown on the Y-axis and amplicon size, in base pairs, 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 allelic imbalance for matched normal (A) or tumor (B) tissue.

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 in16 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 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 upon 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.

Figure 3. Frequency of allelic imbalance in normal and tumor tissues. The numbers of sites of allelic imbalance (i.e. 0, 1, ≥2) were determined in 118 samples of normal tissue and in 239 samples of tumor tissue. The number of specimens in each tissue set (n) is indicated below the set designation. Abbreviations: Lymph Node: LN; Peripheral Blood Lymphocytes: PBL; Acute Myelogenous Leukemia: AML; Chronic Myelogenous Leukemia: CML; Endometrial: Endo. See Materials and Methods for additional details.

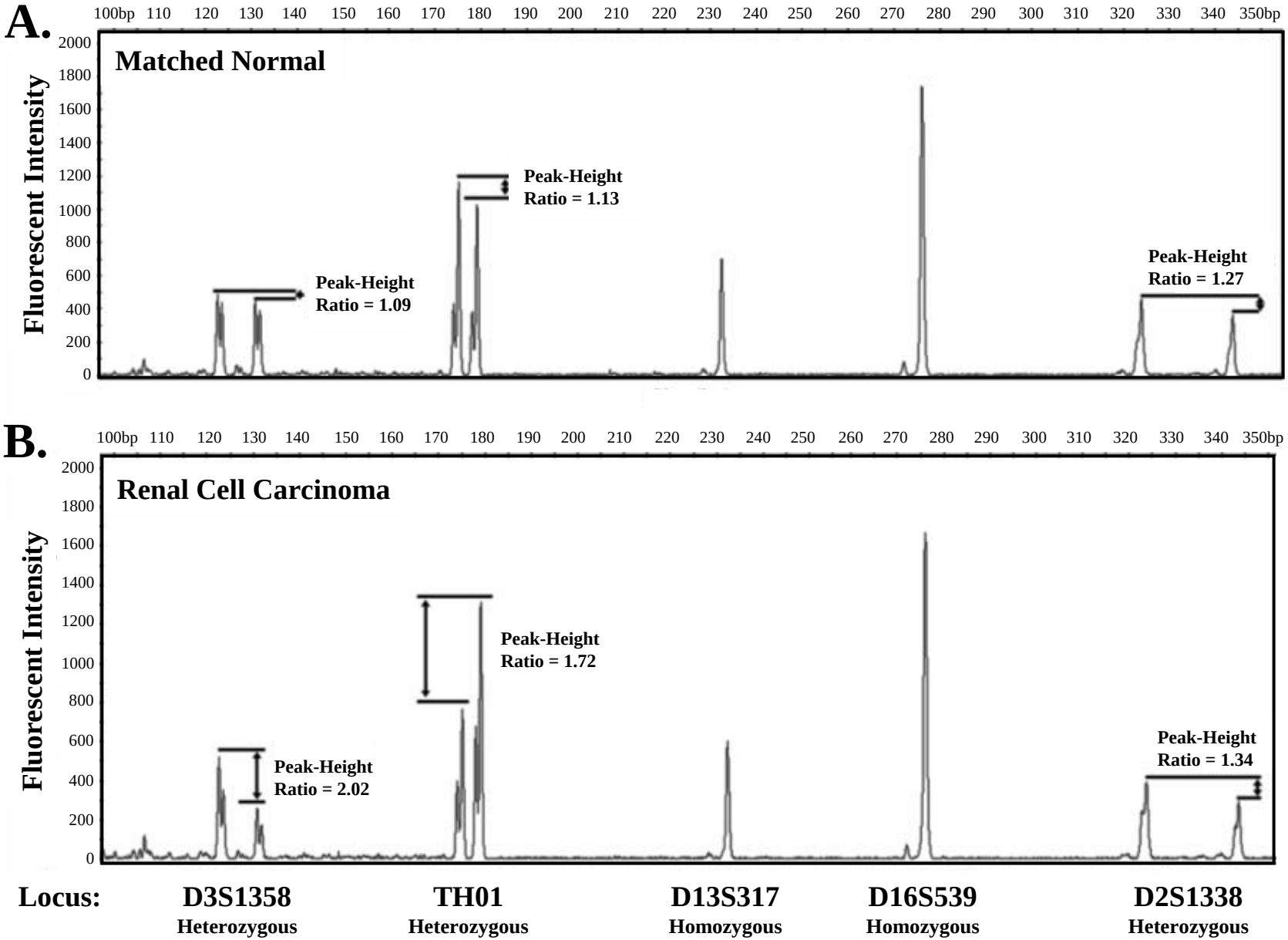
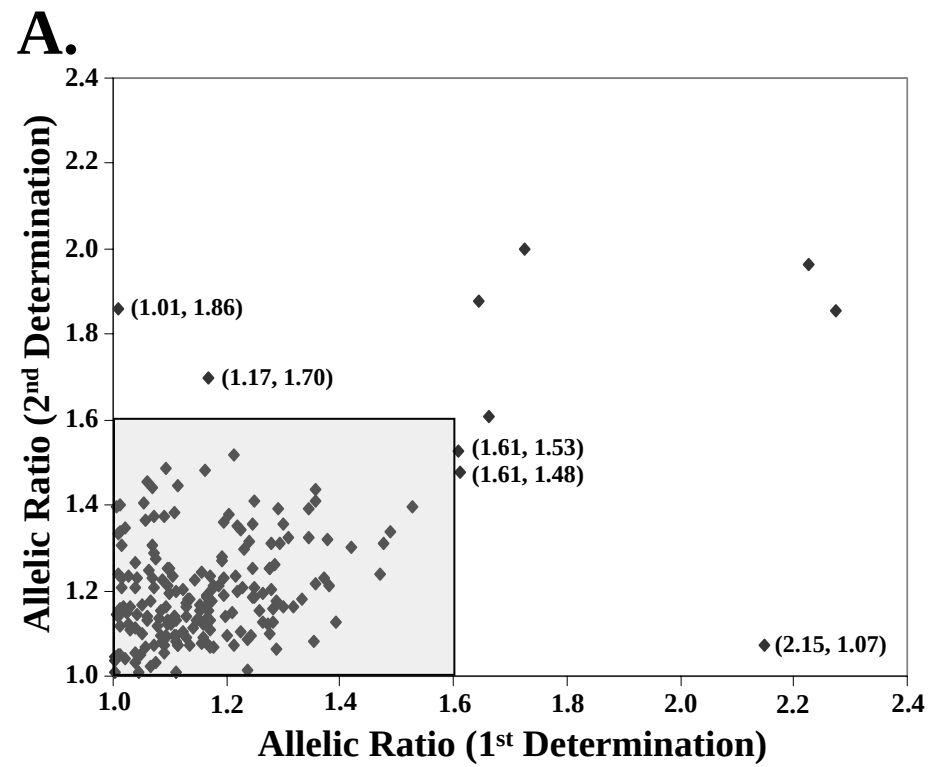

A.
Matched Normal
Fluorescent Intensity
100bp 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350bp
2000 1800 1600 1400 1200 1000 800 600 400 200 0
Peak-Height Ratio = 1.09
Peak-Height Ratio = 1.13
Peak-Height Ratio = 1.27
B.
Renal Cell Carcinoma
Fluorescent Intensity
100bp 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350bp
2000 1800 1600 1400 1200 1000 800 600 400 200 0
Peak-Height Ratio = 2.02
Peak-Height Ratio = 1.72
Peak-Height Ratio = 1.34
Locus: D3S1358 Heterozygous
TH01 Heterozygous
D13S317 Homozygous
D16S539 Homozygous
D2S1338 Heterozygous

Figure 1


A.
Allelic Ratio (2nd Determination)
Allelic Ratio (1st Determination)
(1.01, 1.86)
(1.17, 1.70)
(1.61, 1.53)
(1.61, 1.48)
(2.15, 1.07)
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4

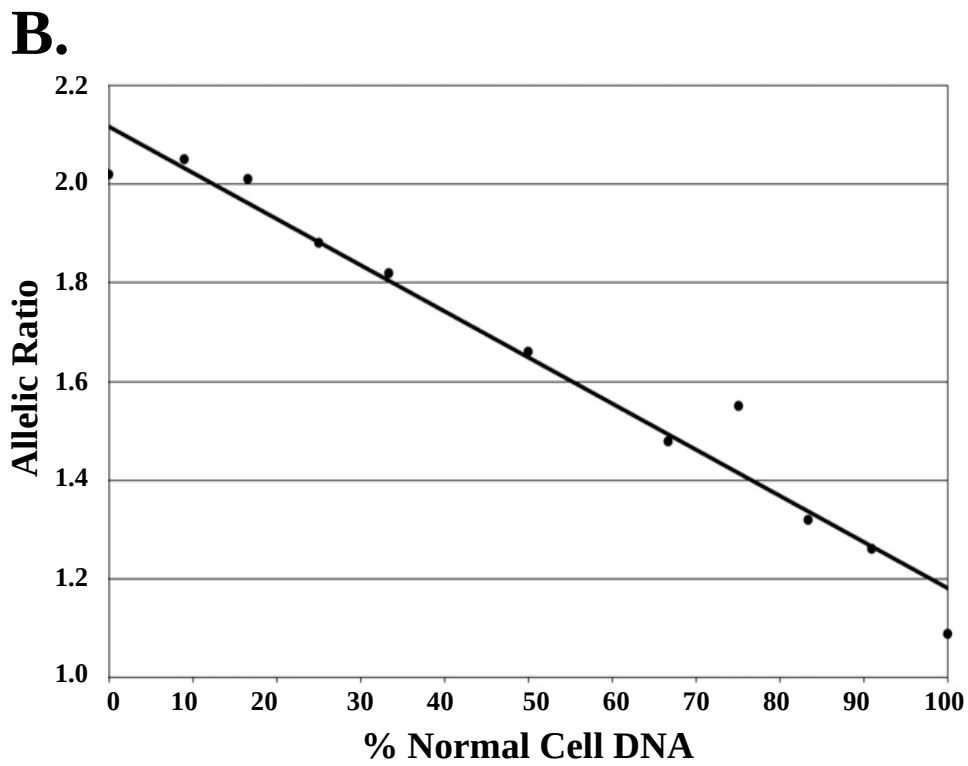

B.
Allelic Ratio
% Normal Cell DNA
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0
10
20
30
40
50
60
70
80
90
100

Figure 2

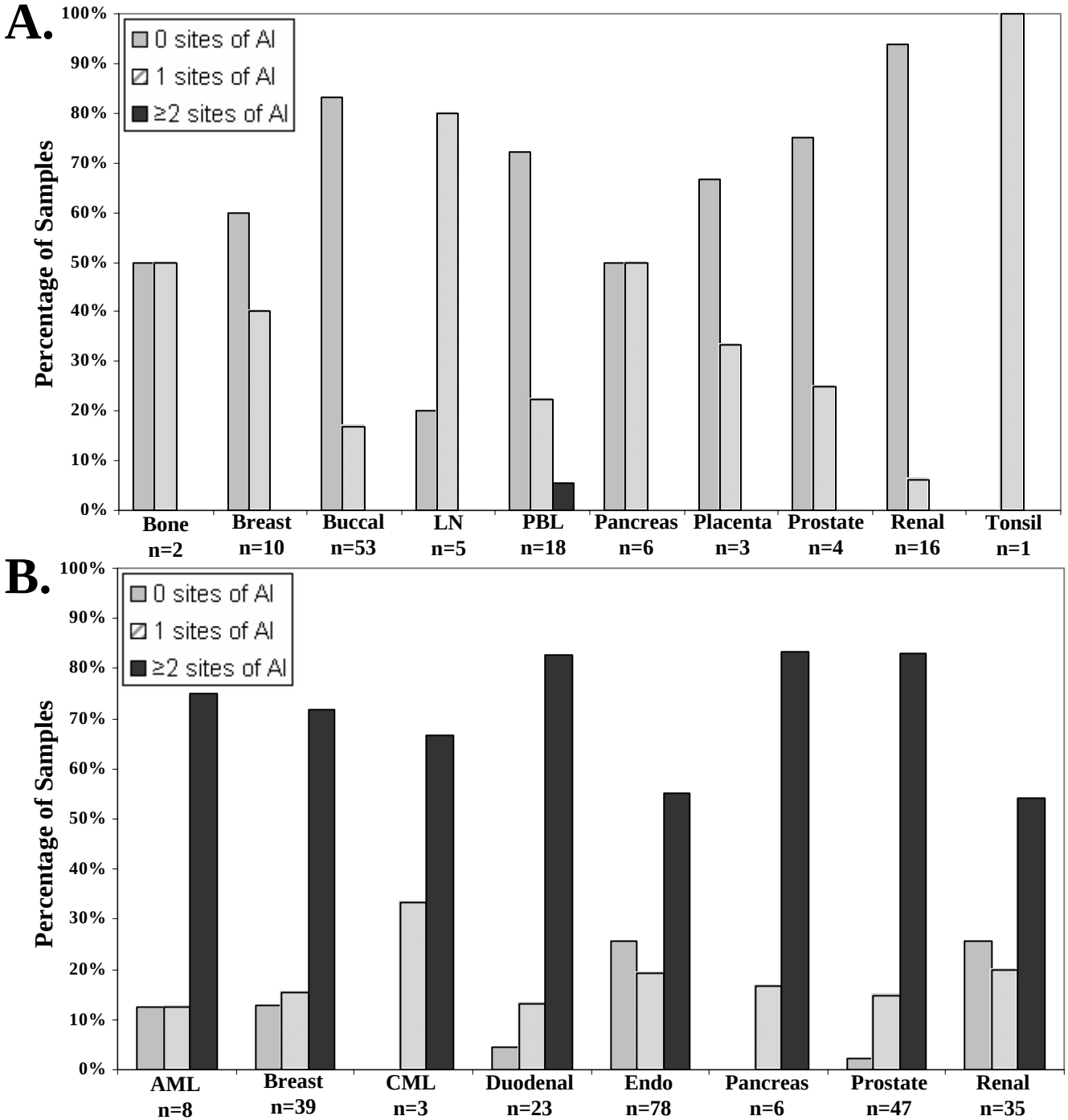

A.
0 sites of AI
1 sites of AI
≥2 sites of AI
Percentage of Samples
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Bone n=2
Breast n=10
Buccal n=53
LN n=5
PBL n=18
Pancreas n=6
Placenta n=3
Prostate n=4
Renal n=16
Tonsil n=1
B.
0 sites of AI
1 sites of AI
≥2 sites of AI
Percentage of Samples
AML n=8
Breast n=39
CML n=3
Duodenal n=23
Endo n=78
Pancreas n=6
Prostate n=47
Renal n=35

Figure 3

APPENDIX D

TELOMERIC DNA CONTENT AND ALLELIC IMBALANCE IN NORMAL, TUMOR-ADJACENT HISTOLOGICALLY NORMAL AND TUMOR PROSTATE TISSUE

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Background: Genomic instability is a source of tumorigenesis and occurs in virtually all tumors, including prostate cancer. Genomic instability can be induced by attrition of telomeres, the structures that stabilize the ends of human chromosomes. Telomeres are partially lost during mitotic DNA replication, which can lead genome-wide allelic imbalance (AI). Markers of genomic instability, such as telomeric DNA content (TC), a surrogate for telomere length, and AI, have previously been shown to have prognostic value in samples sets of prostate and breast cancer patients. This study further evaluates the potential prognostic value of TC and AI. In addition, it addresses the concept of "field effect cancerization", which supports the hypothesis that tumors arise in fields of structurally normal, yet genetically altered cells. It thus attempts to define parameters of true genetic and cellular normalcy within tumors and their surrounding tissues.
Methods: The present study includes a total of 20 tumor samples and matched histologically normal (HN), tumor-adjacent human prostatic tissues (n=40), removed at unknown distances from the tumor margins. These samples were selected based on their biochemical recurrence status, as determined by prostate specific antigen (PSA). Also, two human normal prostatic tissues, confirmed to be disease-free, were included. So far, we have quantitatively measured TC in 12 tumors and their matched HN tissues using a chemiluminescence slot blot titration assay. AI was assessed in 18 tumors, 19 HN tissues, and in the 2 normal, disease-free prostatic tissues using a polymerase chain reaction-based assay that measures genome-wide imbalance of short microsatellite tandem repeats. Associations between TC and AI, and between TC/AI with tissue type were calculated using the non-parametric Wilcoxon-Kruskal Wallis Rank Sums test.
Results: The TC values in the tumors ranged from 0.78 to 2.0 (mean=1.6). The TC values in the HN tissues ranged from 0.89 to 2.5 (mean=1.2). The TC values in tumor tissue are 0.95 to 1.1 (mean=1.0). There was no significant difference between TC in tumor and HN tissues. AI in the tumor and in the HN tissues ranged from 1.0 to 7.0 affected genomic sites (mean=2.5), and from 1.0 to 8.0 affected genomic sites (mean=2.6), respectively. The normal disease-free tissues had no affected genomic sites. There was no significant association between TC and AI in these samples. AI was not associated with biochemical recurrence; however, a trend was observed between the latter and TC.
Conclusions: The extent of AI in the normal disease-free tissues was substantially lower compared to AI in the tumor and HN tissues. In contrast, AI and TC were similar in tumor and HN tissues. Our results support the concepts of "field cancerization" and "cancer field effect," i.e areas within tissues consisting of structurally normal, yet genetically aberrant, cells that represent fertile grounds for tumorigenesis. The latter has clinical implications for tumor margin and surgical assessment.

APPENDIX E

TELOMERE DNA CONTENT AND ALLELIC IMBALANCE IN HISTOLOGICALLY NORMAL TISSUE ADJACENT TO BREAST TUMORS

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Background: Genomic instability is important to the pathogenesis of malignant transformation in human cancers. However, the precise molecular mechanisms underlying chromosomal rearrangements remain largely unknown. One mechanism leading to genomic instability is loss of telomere function. Telomeres are specialized nucleoprotein complexes that protect the ends of chromosomes from degradation and end-to-end fusion, events that spur chromosomal aberrations. Telomere attrition, induced by incomplete DNA replication during mitosis, is an early occurring event of cancers, including breast cancer. In addition, telomere attrition and other measures of genomic instability, have been shown to occur in histologically normal tissue adjacent to breast tumors.
Study Purposes: (A) To identify fields of histologically normal, yet genetically unstable cells within breast tissues that support breast tumorigenesis. For this, we measured markers of genomic instability, such as telomeric DNA content (TC) and allelic imbalance (AI) at defined distances from breast tumor margins. (B) To test the hypothesis that TC correlates with breast cancer progression. This is implied from the fact that genomic instability leads to the development of cells with invasive and metastasizing characteristics, i.e. cells responsible for disease recurrence.
Material and Methods: The present study included (i) breast tumors (n=11) with matched histologically normal tissues excised at 1 cm and 5 cm from the visible tumor margins; (ii) a case-control group consisting of large, node-positive breast tumors (n=25); for this group, proximal histologically normal tissue was also available. TC was measured using a chemiluminescence hybridization assay, and AI was determined by multiplex PCR analysis of 16 genome-wide unlinked microsatellite loci. Associations between TC/AI and site of tissue were analyzed by Wilcoxon/Kruskal Wallis Rank Sums test; associations between TC and time of disease-free survival were determined by Kaplan/Meier Log Rank analysis.
Results: TC and AI differed as a function of distance from the tumor margin ($p=0.0003$ and $p=0.0066$, respectively). TC in proximal histologically normal tissues resembled TC of tumors, rather than TC of normal. In the case-control group, TC was associated with disease recurrence within 84 months of surgery ($p=0.012$) and with time of disease-free survival ($p=0.017$). TC was also associated with recurrence and time of disease-free survival in proximal histologically normal tissue ($p=0.025$ and $p=0.006$).
Conclusions: Our data indicate that telomere attrition and allelic imbalance, two markers of genomic instability, occur in areas of histologically normal tissues adjacent to breast tumors. These areas may represent fertile grounds for the malignant transformation of epithelial cells. Our study also shows that TC is a prognostic marker of disease recurrence in breast cancer. Thus,

TC has the potential to identify breast cancer patients at high risk for disease progression and could be used in the clinical management of breast cancer.

APPENDIX F

ASSOCIATION BETWEEN CANCER-FREE SURVIVAL AND TELOMERE DNA CONTENT IN PROSTATE TUMORS

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Purpose: To evaluate the hypothesis that telomere DNA content (TC) in prostate tumor tissue is associated with time to prostate cancer recurrence.
Materials and Methods: The cohort was comprised of 77 men who had prostatectomies between 1982 and 1995. Slot blot assay was used to measure TC in DNA extracted from paraffin-embedded tumor and nearby histologically-normal prostate (NHN) tissues. Multivariable Cox proportional hazards analysis related TC, patient age at diagnosis, Gleason sum and pelvic node involvement to the time of prostate cancer recurrence. Regression analysis related TC in paired tumor and NHN tissues. Nonparametric Kruskal-Wallis analysis related TC in tumor and NHN tissues with 72-month disease free survival.
Results: TC was a predictor of time to prostate cancer recurrence controlling for age at diagnosis, Gleason sum, and pelvic node involvement (RH= 5.02, 95% CI 1.40-17.96, p = 0.0132). TC in tumor tissue was associated with TC in NHN tissue (R=0.601, p<0.0001). Median TC in both tumor and NHN tissues from men whose cancer recurred within six years was approximately half that in men who remained disease-free (p=0.012, p=0.024, respectively).
Conclusions: Diminished TC in prostate tissues obtained at radical prostatectomy predicts prostate cancer recurrence, independent of age at diagnosis, Gleason sum, and pelvic node involvement. TC in tumor tissue is also associated with TC in NHN prostate tissue. Thus, mechanisms known to generate genomic instability are operative in fields of cells beyond the tumor margins, prior to histological changes.

APPENDIX G

TELOMERE DNA CONTENT AND ALLELIC IMBALANCE PREDICT DISEASE-FREE SURVIVAL AND DEFINE FIELD CANCERIZATION IN HISTOLOGICALLY NORMAL TISSUE ADJACENT TO BREAST TUMORS

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Purpose: Cancer arises from an accumulation of mutations that promote the selection of cells with increasingly malignant phenotypes. Telomere attrition and other measures of genomic instability have been shown to occur in histologically normal tissue adjacent to breast tumors. The purpose of this study was to (i) test the hypothesis that telomere DNA content (TC) correlates with staging and breast cancer progression and (ii) identify fields of histologically normal, yet genetically unstable cells within breast tissues, which support breast tumorigenesis, by measuring TC and allelic imbalance (AI), markers of genomic instability, at defined distances from breast tumor margins.
Methods: Four independent sets of human breast tissues were used in this study. The first set (’82-’93) was comprised of 77 archival frozen and paraffin-embedded breast tumor tissues from women diagnosed with either invasive ductal or lobular carcinomas between 1982 and 1993. The second set (’96-’99) was comprised of 63 archival paraffin-embedded breast tissues from women diagnosed with ductal carcinoma in situ (DCIS), invasive ductal carcinomas or invasive lobular carcinomas between 1996 and 1999. The third set was comprised of 12 breast tumors with matched tumor-adjacent, histologically normal (TA-HN) tissues excised at 1 cm and 5 cm from the visible tumor margins. The fourth set was comprised of 20 normal, disease-free breast tissue (RM) obtained from women who have undergone reduction mammoplasty. TC was measured using a chemiluminescence hybridization assay and AI was determined by multiplex PCR analysis of 16 genome-wide unlinked microsatellite loci. Associations between TC and time of disease-free survival were determined by Kaplan/Meier Log Rank analysis; whereas, other associations were analyzed by either Wilcoxon/Kruskal Wallis Rank Sums test or logistical regression.
Results: In the current investigation we have defined a normal range of TC (75-143% of the placental DNA standard) using breast and other tissues from multiple sites in healthy donors, compared this range to the distribution of TC measured in breast tumor tissues, and used this information to evaluate the relationships in breast tumor tissues between TC and tumor size ($p=0.03$), nodal involvement ($p<0.0001$), TNM stage ($p=0.002$), overall five-year survival ($p=0.0001$), five-year breast cancer survival ($p=0.0004$), and breast cancer-free survival interval following surgical excision of breast carcinoma ($p=0.013$). In addition, we have measured TC and AI at defined distances from breast tumor margins. Tissues from the third set showed TC and AI differed as a function of distance from the tumor margin ($p<0.001$ and $p<0.01$, respectively).
Conclusions: Our study shows that TC is a prognostic marker of disease recurrence in breast cancer and may be able to provide staging information at time of biopsy. In addition, our data indicate that telomere attrition and allelic imbalance occur in histologically normal areas of breast tissues, which is in agreement with the concept of “cancer field effect”.