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14. ABSTRACT

We are using an innovative, quantitative assay for telomere DNA content (TC) developed and characterized by the PI, to test the hypothesis that TC predicts the likelihood of disease recurrence in women with DCIS. In Year One, we collaborated to determine whether TC measured in bulk DCIS tumor tissue is comparable to that measured in tumor epithelial cells purified by laser-capture microscopy. In 7/10 instances, TC in microdissected specimens was 72-112% of that in the undissected control (mean 85%). These results suggest that it will not be necessary to microdissect or otherwise fractionate DCIS specimens prior to TC analysis. We are currently confirming and extending these results in our own laboratory. In Years One and Two, we measured TC in 46 specimens of normal breast tissue, 126 specimens of DCIS and 657 specimens of breast tumor tissues. In Year Two, we used a Kaplan-Meier plot and log-rank test to show that low TC predicts a shorter survival interval. TC was not associated with ethnicity, menopausal status, or the expression of several other markers, including estrogen receptor, progesterone receptor, p53, Ki67, and Her2. We are working with the New Mexico Tumor Registry to identify additional specimens of DCIS from women with longer follow-up to confirm and extend these results. In summary, during Years One and Two we have shown that (i) meaningful TC measurements can be obtained with bulk DCIS tissues, (ii) TC is associated with tumor stage and (iii) TC in DCIS is associated with breast cancer-free survival.

15. SUBJECT TERMS
Ductal carcinoma in situ (DCIS), breast cancer, telomeres, prognosis, genomic instability

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Introduction

The widespread use of screening mammography has resulted in a ten fold increase in the incidence of ductal carcinoma in situ (DCIS) over the past twenty years, from 4800 cases in 1983 to more than 50,000 cases in the US in 2003 (1-3). This accounted for 18.3% of all newly diagnosed breast tumors, and 23% of newly diagnosed breast tumors in women 40-49 years of age in 2003 (2,3). However, the fraction of women with DCIS who eventually progress to invasive breast cancer is small (4-6). A Danish autopsy study found that 25% of women had *in situ* carcinomas, including DCIS, at their death, although the lifetime risk of developing breast cancer during the same period was only 1% (7). Similarly, only 32% of women whose DCIS was misdiagnosed as normal, and so did not receive treatment, went on to develop invasive carcinoma within 30 years of their biopsy (8,9). Less than 2% of women with DCIS die from breast cancer within ten years of diagnosis (10). Taken together, these results imply that up to two-thirds of women with DCIS would not progress to invasive cancer, even without treatment. Unfortunately, currently available prognostic markers are unable to discriminate between DCIS that will and will not progress, so many women receive aggressive treatments that may be unnecessary. Currently, 97.5% of women with DCIS in 1999 had some type of surgery, of which 28% had radical mastectomies (11). In the March, 2004 issue of the Journal of the National Cancer Institute, Baxter et al (11) wrote: "*The potential for preventing invasive breast cancer is important, yet the risk for over treatment is a clinically significant concern*". In an accompanying editorial, Dr. Morrow comments on barriers to developing meaningful therapeutic guidelines (12). She writes:

"*The first of these is our inability to identify which DCIS lesions will progress to invasive carcinoma, and in what time interval. Conventional prognostic factors, such as patient age and tumor grade, subtype, and size, provide information on the time course of local recurrence and the magnitude of risk reduction achieved with radiotherapy, but these factors do not identify those women who will have a disease recurrence with potentially life-threatening invasive cancer. Efforts to identify a molecular signature for DCIS lesions that will recur as invasive carcinoma are of enormous interest…*"

Using an innovative, quantitative assay for telomere DNA content (TC) developed and characterized by the PI (13-17), we have recently shown that TC in tumor tissue is associated with cancer-free survival in women with breast cancer (18). The purpose of this investigation is to determine whether TC can be used similarly to predict the likelihood of disease progression in women with DCIS.

Body

Tasks: The agreed upon tasks to be completed during the first year of the IDEA Award were:

Aim One: We will compare TC measured in bulk DCIS tumor tissue to TC measured in tumor epithelial cells that have been stripped of stromal cells and connective tissue by laser-capture microscopy.

Task 1	Months 1-6	Obtain 30 random archival specimens of DCIS.
Task 2	Months 2-12	Extract DNA from bulk DCIS samples and measure telomere DNA content (TC). Divide study group into thirds, based on TC.
Task 3	Months 4-12	Use laser capture microscopy to purify tumor epithelial cells from stromal and cells and connective tissues in the 10 samples comprising the middle third of the study group.
Task 4	Months 6-12	Extract DNA from purified epithelial cells and measure TC. Compare TC in bulk DCIS tissue to purified tumor epithelial cells.

Aim Two: The data from aim one will be used to guide the study design for the second aim, in which we will perform a retrospective study of the association between TC and time to disease recurrence in women with DCIS.

Task 5 Months 1-6 Design search parameters for NMTR database and identify 120 members of study group.
Task 6 Months 1-6 Establish data base of patient records
Task 7 Months 3-24 Obtain tissue blocks and cut new sections.
Task 8 Months 12-30 Extract DNA from 120 bulk specimens of DCIS or enriched epithelial cells, depending on the outcome of Aim One, and measure TC.

Progress Relative to Tasks:

Tasks 1 and 2 have been completed. Retrospective DCIS cases (N=27) were obtained from the Surgical Pathology Department at the University of New Mexico Hospital (UNMH). All cases were diagnosed in 2004 and 2005 and the presence of DCIS was confirmed by Dr. Nancy Joste, Director of Anatomic and Cyto-pathology. Four 25 µm sections of the paraffin-embedded, formalin-fixed (FFPE) tissue were obtained and DNA was isolated using standard techniques. TC was analyzed for all the samples and the TC distribution of the samples is plotted in Figure 1. TC in these samples ranged from 58-218% of the placental DNA control with a mean TC value of 145%.

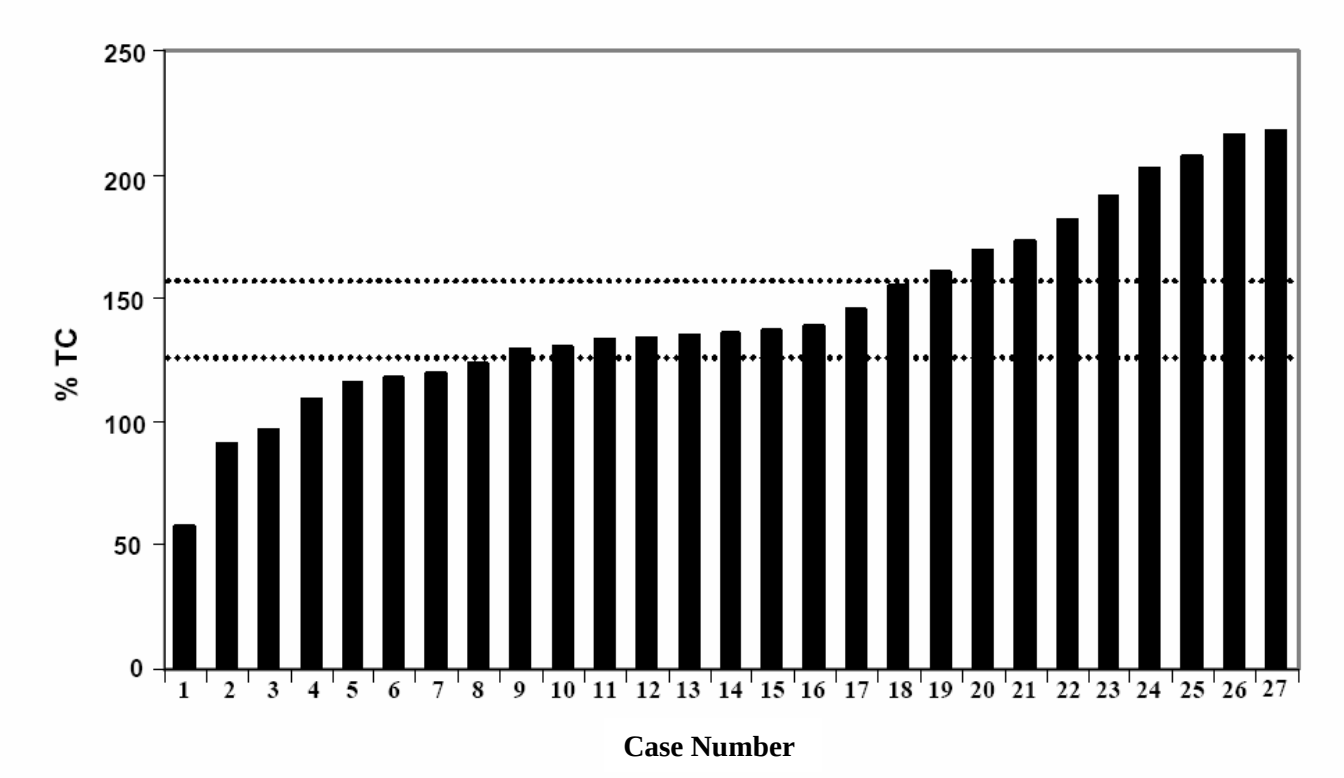

Figure 1. Distribution of Telomere DNA Contents (TC) in 27 Specimens of Bulk DCIS tissues. Each DCIS specimen is shown on the x-axis and TC (expressed as a percentage of TC in the placental DNA control) is shown on the y-axis. The two dotted lines represent the upper (155%) and lower (129%) limits for the middle 10 cases used in subsequent analyses.

Tasks 3 and 4 are partially complete. To minimize possible delays resulting from tissue acquisition, we collaborated with Dr. Colleen Fordyce in Year One to measure TC in 10 pairs of bulk DCIS tissue obtained from her laboratory (Figure 2). In 7/10 instances, TC in the microdissected specimens was 72-112% of that in the undissected control. The difference in TC in bulk and microdisected tissue was relatively constant (median 85%), implying that it would not be necessary to microdissect or otherwise fractionate DCIS specimens prior to TC analysis.

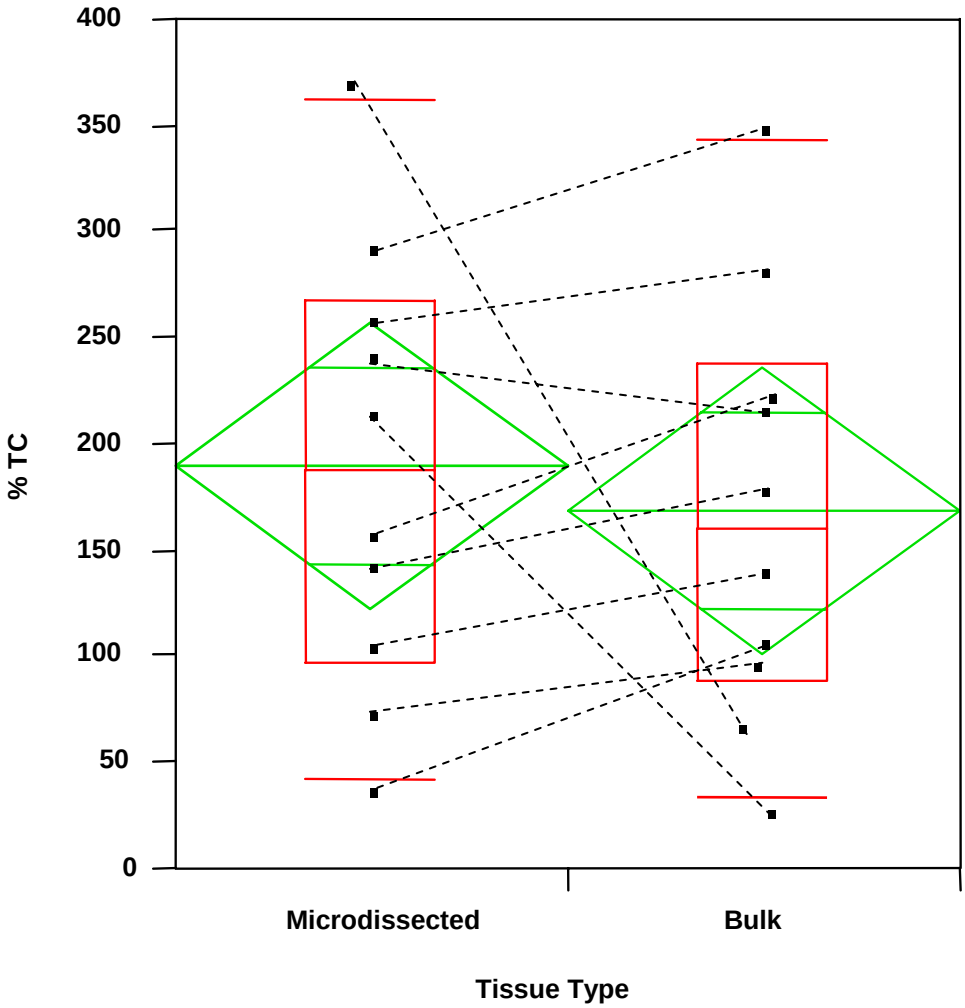

Figure 2. Distributions of Telomere DNA Contents (TC) in Ten Pairs of Microdissected and Bulk DCIS Tissues. TC is shown on the y-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are shown as lines above and below the box. The line across each diamond represents the group mean. The height of each diamond represents the 95% confidence interval for each group.

In Year Two, we obtained and characterized DCIS tissues that will allow us to confirm these results in our own laboratory. The 10 cases of DCIS from Figure 1 that comprise the middle tertile of TC values (Range: 129-155% of the placental DNA control) were further analyzed. Ten consecutive 10 µm sections were obtained for these samples with the odd numbered sections fixed to slides and the even numbered sections pooled together for bulk TC analysis. The samples for the bulk TC were again analyzed and compared to the original TC values. The results are shown in Figure 3. TC in the initial bulk analysis and the subsequent bulk analysis was not statistically significantly different (p=0.762) and, in fact, yielded nearly identical results (median 98%). In addition, members of the laboratory received hands-on training for Laser Capture Microscopy (LCM) using the Arcturus PixCell II LCM Workstation. Lab members were trained to use the proper procedures to microdissect areas of DCIS and subsequently isolate DNA for TC analysis from formalin-fixed, paraffin-embedded tissue sections. We are currently using LCM to isolate only cells with DCIS from the odd numbered sections described in Figure 3, so as to confirm, independently, the results shown in Figure 2.

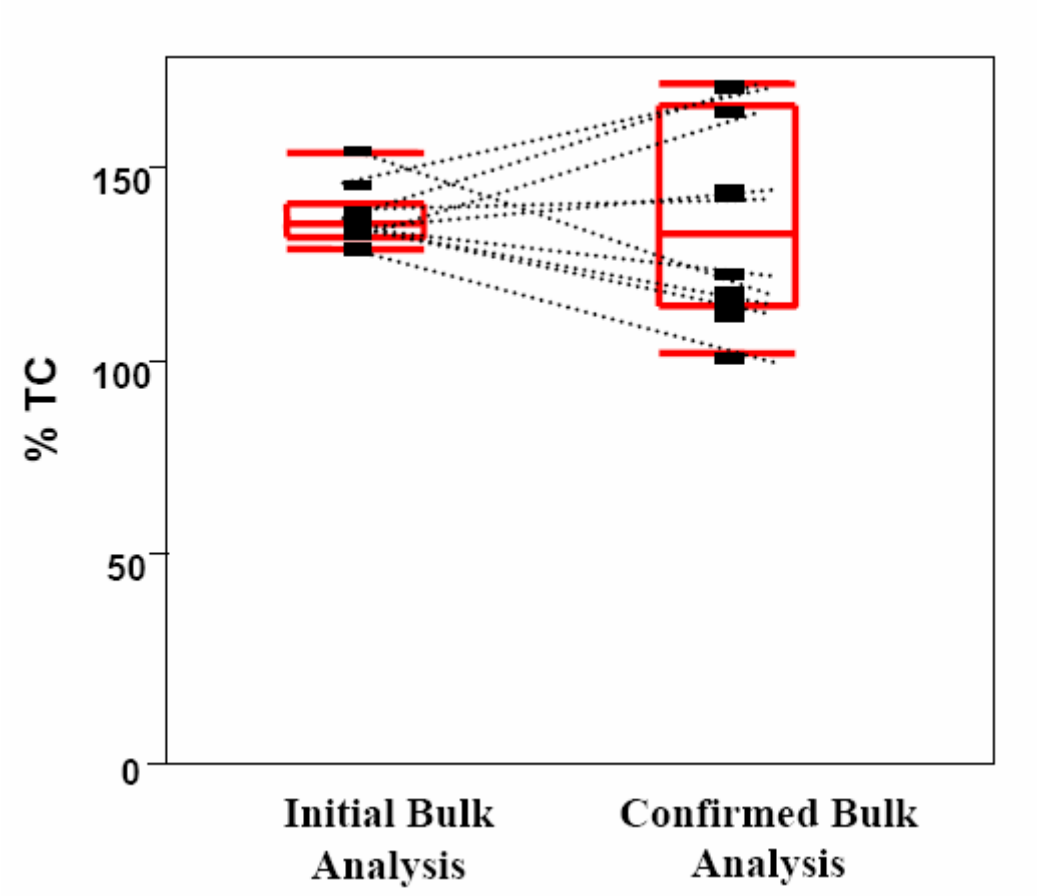

Figure 3. Distributions of Telomere DNA Contents (TC) in 10 Specimens of DCIS Tissues. TC (expressed as a percentage of TC in the placental DNA control) is shown on the y-axis. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are shown as lines above and below the box. Data points for each sample are connected by a dotted line.

Tasks 5 and 6 have been completed.

Tasks 7 and 8 are ongoing. In Years One and Two, we purified DNA and measured TC from 126 specimens of DCIS. For comparison, we also measured TC in 46 specimens of normal breast tissue obtained from reduction mammoplasty, and 657 specimens of breast cancer tissues TNM stages I-IIIA (Figure 4). Non-parametric Rank Sums (Kruskal-Wallis) test demonstrates a statistically significant difference between the mean TC in DCIS tissue and all TNM stages of breast cancer tissues ($p<0.0001$ for each).

A large fraction of the samples shown in Figure 4 (N=530) was collected as a part of the prospective, population-based Health, Eating, Activity and Lifestyle (HEAL) Study. The HEAL multi-center study was designed to evaluate the association between body composition, hormones, diet, physical activity, and prognosis over time for non-Hispanic white, Hispanic, and African-American women ascertained through the Surveillance, Epidemiology, and End Results (SEER) registries. In Year Two, we completed TC analysis and retrieved coded patient data, stripped of all personal identifiers, as approved by the University of New Mexico Human Research Review Committee. The cohort was initially divided into sixths, the survival interval for each group was calculated, and the results were evaluated for statistical significance by log-rank analysis. Groups with statistically indistinguishable survival intervals were combined and the process was repeated until only groups with significantly different survival intervals remained. Using this process, the cohort was stratified into two TC groups: low TC

was defined as ≤ 200% in the placental DNA control, and high TC was defined as > 200% of TC in the placental DNA control, respectively.

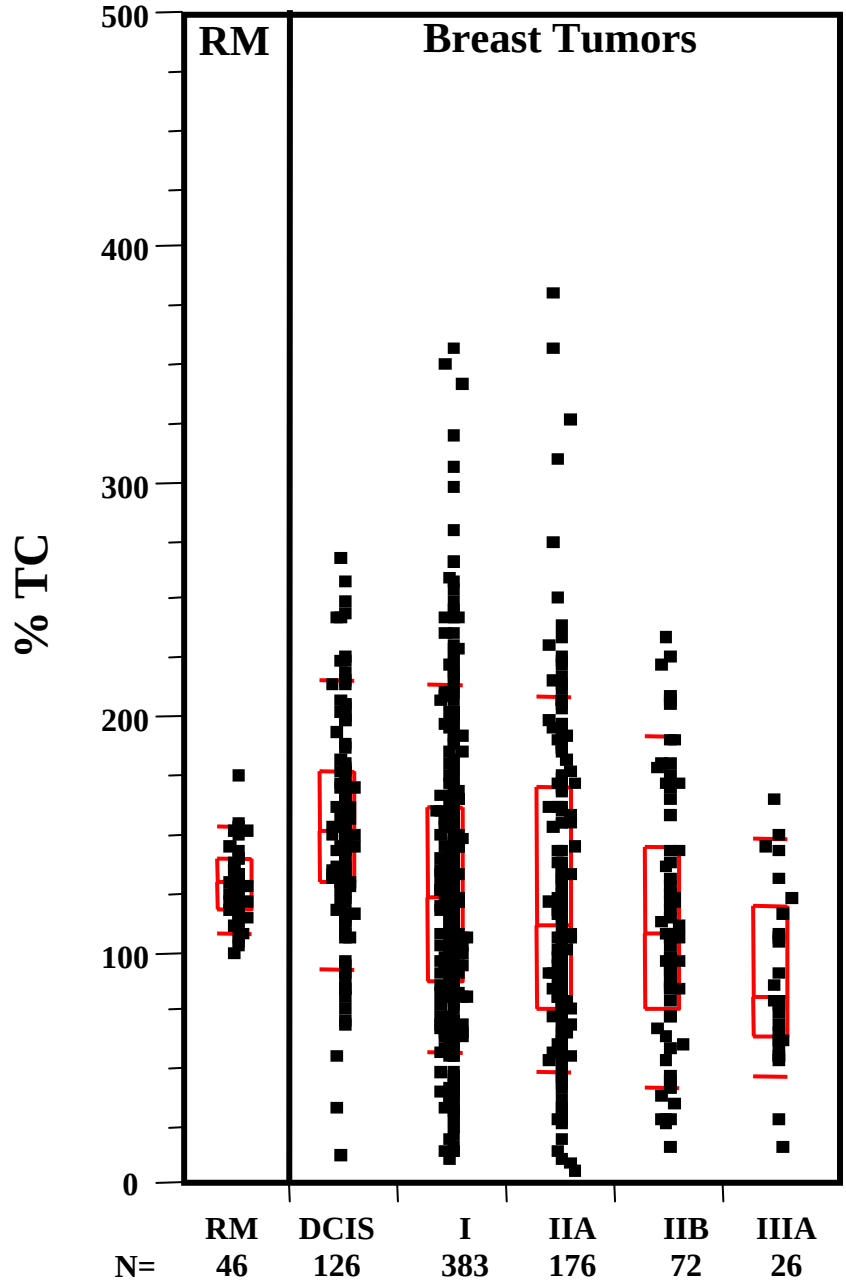

Figure 4. Distributions of Telomere DNA Contents (TC) in Normal Breast Tissues, DCIS, and Invasive Breast Tumors. TC is shown on the y-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (N) is indicated below the set designation on the x-axis. The 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.

We next extracted the data for the subset of the HEAL cohort with DCIS (N=97). The mean age and follow-up of cohort members were 57.1 (Range: 36-89; SD: 11.7) and 6.9 (Range: 2.8-9.0; SD: 1.0) years, respectively. At the time of analysis, 96% of the cohort members were alive. Additionally, 89% of the cohort members were free of disease, either at time of analysis or at time of their non-breast cancer related death. We evaluated the prognostic value of the low (N=80) and high (N=17) TC groups in predicting breast cancer-related, adverse event-free survival interval. An adverse event was defined as death due to breast cancer, breast cancer recurrence or development of a new primary breast tumor.

Eleven breast cancer-related adverse events had occurred by the time of the analysis. A Kaplan-Meier plot and log-rank test (Figure 5) demonstrated a trend, although not statistically significant, showing low TC predicts a shorter survival interval (p=0.111). All of the 11 cases with a documented adverse event were in the low TC group. TC was not associated with ethnicity, menopausal status, or the expression of several other markers, including estrogen receptor, progesterone receptor, p53, Ki67, and Her2.

The data obtained to date, while not yet statistically significant, are consistent with the hypothesis that high TC predicts breast cancer-free survival in women with DCIS. The New Mexico Tumor Registry is currently working to identify additional specimens of DCIS from women with longer follow up, including as many as possible who had recurrent disease, to extend these results.

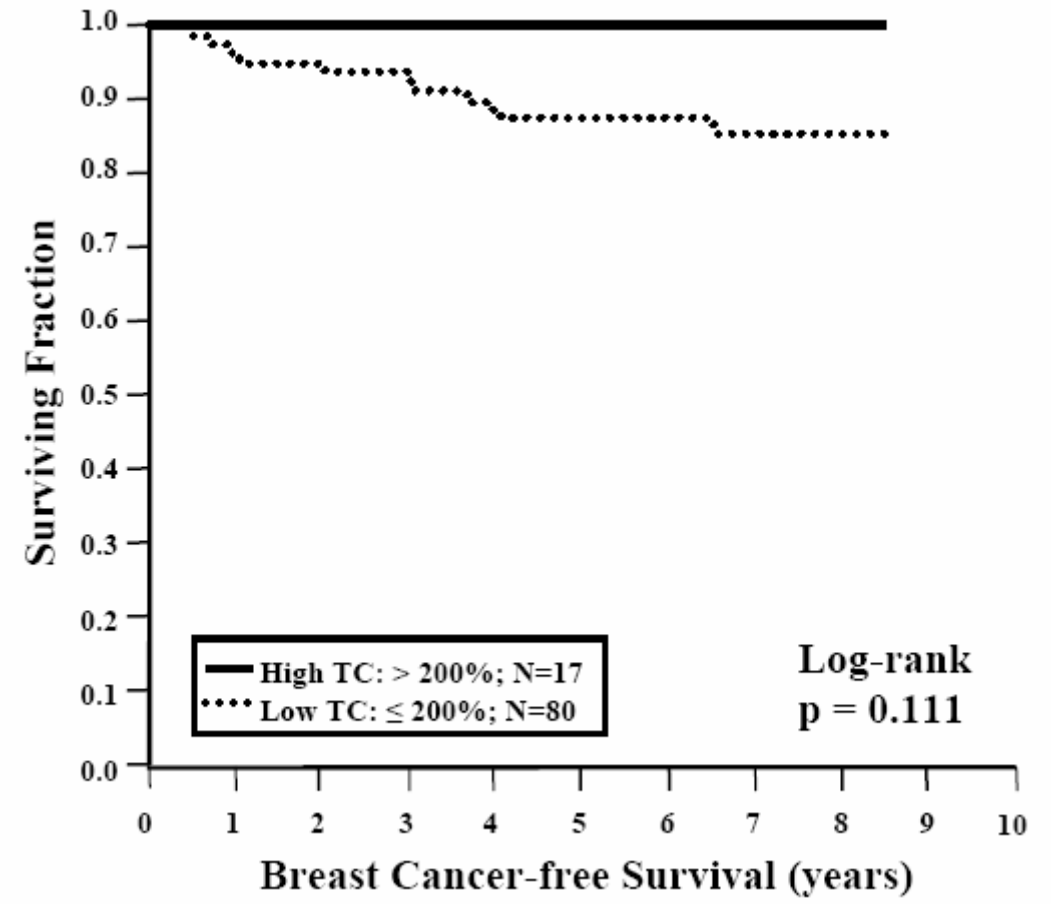

Figure 5. Breast Cancer-free Survival Interval by Telomere DNA Content in 97 DCIS cases. The set of DCIS cases was divided into two groups based on the low (N=80) and high (N=17) TC cutoff (200% of placental DNA control). Breast cancer-free survival interval in years, is shown on the x-axis. The surviving fraction is shown on the y-axis. Subjects were censored at the time lost to follow-up. The log-rank test was used to test the significance (p) of the differences in the group's survival intervals. N represents the number of subjects in each group.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR TWO

- Telomere DNA content has been analyzed in 46 specimens normal breast tissue, 126 specimens of DCIS and 657 specimens of breast tumor tissues.
- Telomere DNA content was compared in 27 specimens of bulk DCIS tissue, pooled DCIS tissues from serial slides, and individual slides that will be used for Laser Capture Microscopy.
- Lab members were trained to use the proper procedures to microdissect areas of DCIS and subsequently isolate DNA for TC analysis from formalin-fixed, paraffin-embedded tissue sections.

- Additional tissue procurement is ongoing.
- Clinical and patient data was obtained for 97 members of HEAL Cohort.
- Breast cancer-free survival data in women with DCIS was analyzed as a function of telomere DNA content.

REPORTABLE OUTCOMES FOR YEAR TWO

- A database has been produced that contains anonymous patient histories, including age at diagnosis, ethnicity, treatments, tumor stage, estrogen and progesterone receptor status, tumor size, length of disease-free survival or date and cause of death and diagnosis.
- DNA banks from 46 specimens of normal breast tissue, 126 specimens of DCIS and 657 specimens of breast tumor tissues have been produced.
- Data from this investigation is included in one published (18, appendix), and one submitted (19) manuscript.

CONCLUSIONS

- Prior studies show that telomere DNA content (TC) is a novel and independent prognostic marker in breast tumors. The data obtained thus far are consistent with the conclusions that: (i) meaningful TC measurements can be obtained with bulk DCIS tissues, (ii) TC is associated with tumor stage and (iii) TC in DCIS is associated with breast cancer-free survival.

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Preclinical study

Telomere content correlates with stage and prognosis in breast cancer

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

Summary

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

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

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

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

Introduction

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

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

[†]Colleen A. Fordyce and Christopher M. Heaphy contributed equally to this study.

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

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

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

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

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

Materials and methods

Tissue samples

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

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

Table 1. Characteristics of tumor tissues

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

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

[a]Tissue sets are described in the Materials and methods section.

[b]Of the 10 subjects who died from causes other than breast cancer, six died from other cancers, and one each from dementia, hypertension, pulmonary embolism, and unknown causes.

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

Histological review

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

Determination of telomere DNA content (TC)

DNA was extracted from slides cut from frozen or paraffin-embedded tissue, and TC was measured as described [20,26]. Briefly, DNA was isolated from frozen or paraffin-embedded tissues, and blood samples using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56 °C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5′-TTAG GG-3′)$_4$-FAM, (IDT, Coralville, IA) was hybridized to the genomic DNA, and the membranes were washed to remove non-hybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm for 2–10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal from three replicate determinations of each tumor DNA relative to the average chemiluminescent signal in the same amount (typically 20 ng) of a reference DNA standard (placental DNA). DNA purified from HeLa cells, which have approximately 30% of the TC in placental DNA, and samples prepared without DNA served as positive and negative controls, respectively.

Statistical methods

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

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

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

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

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

Results

Telomere contents in normal tissues

Telomere content can be affected by several inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To evaluate the potential variability in TC arising from inherent properties of tissues, TC was measured in a diverse sampling of 70 specimens of normal tissue from multiple organ sites (Figure 1). Specimens included breast tissue obtained by reduction mammoplasty (RM); histologically normal breast tissues excised from sites 5 cm from the breast tumor margins (HNB), buccal cells from healthy, young men and women (BUC) and PBL from women with a prior diagnosis of breast cancer (PBL). As summarized in Table 2, median TC in HNB and PBL sets (101 and 87%, respectively) were approximately 30% lower than median TC in the RM and buccal specimens (126 and 110%, respectively). Similarly, the median ages for the donors of the HNB set (53 years) was almost twice the median ages of the donors of the RM samples (30 years). Although the ages of the volunteers contributing the BUC and PBL samples were not collected, the BUC samples were obtained from college students in their early 20s, while the PBL samples were obtained from a subset of a larger study group with a median age of 58 years. Thus, the results are consistent with the accepted view that telomere length in humans decreases as a function of age [13].

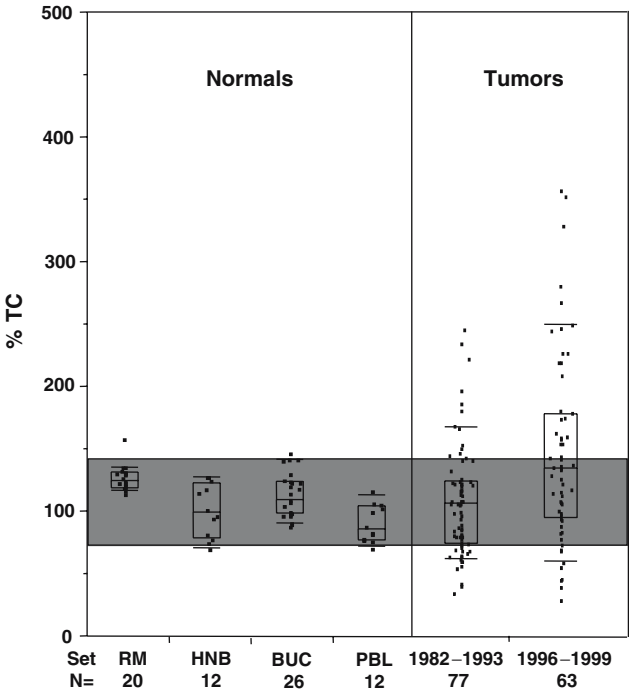

Figure 1. Distributions of telomere DNA contents (TC) in normal and tumor tissues. TC is shown on the *y*-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the *x*-axis.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

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

Telomere contents in breast tumor tissues differ from normal tissues

Matched tumor tissue was available for the 12 specimens of HNB tissues described above. Although TC in 11/12 of the HNB tissues fell within the expected range for normal tissues, only 2/12 matched tumors had TC within this range (Table 3). On average, TC in tumors was 61% of TC in the matched HNB tissues. TC was measured next in the 140 tumors comprising the 1982–1993 and 1996–1999 tumor sets (Figure 1). The IQR for TC in the two sets of tumor tissues, 49 and 79%, respectively, were substantially greater than the 28% IQR of the normal tissues (Table 2). Fifty-six percent of breast cancer specimens in the 1982–1993 set had TC values within the range that contained 95% of normal tissues, while 23 and 21% had TC values less and greater than the normal range, respectively. Similarly, only 43% of breast cancer specimens in the 1996–1999 set had TC values within the range that contained 95% of normal tissues, while 14% were below the range and 43% were above. Thus, TC in breast cancer tissues is significantly more heterogeneous than that in normal tissues, reflecting frequent abnormally short and long telomeres.

Table 3. TC in paired HNB and tumor tissue

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

Additional details are found in the text and the legend to Figure 1. T/N is the percent TC in the tumor (T) relative to TC in the paired, histologically normal (HNB) tissues.
[a]Tissue sets are described in the Materials and methods section.

Telomere contents in breast tumor tissues are associated with TNM stage

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

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

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

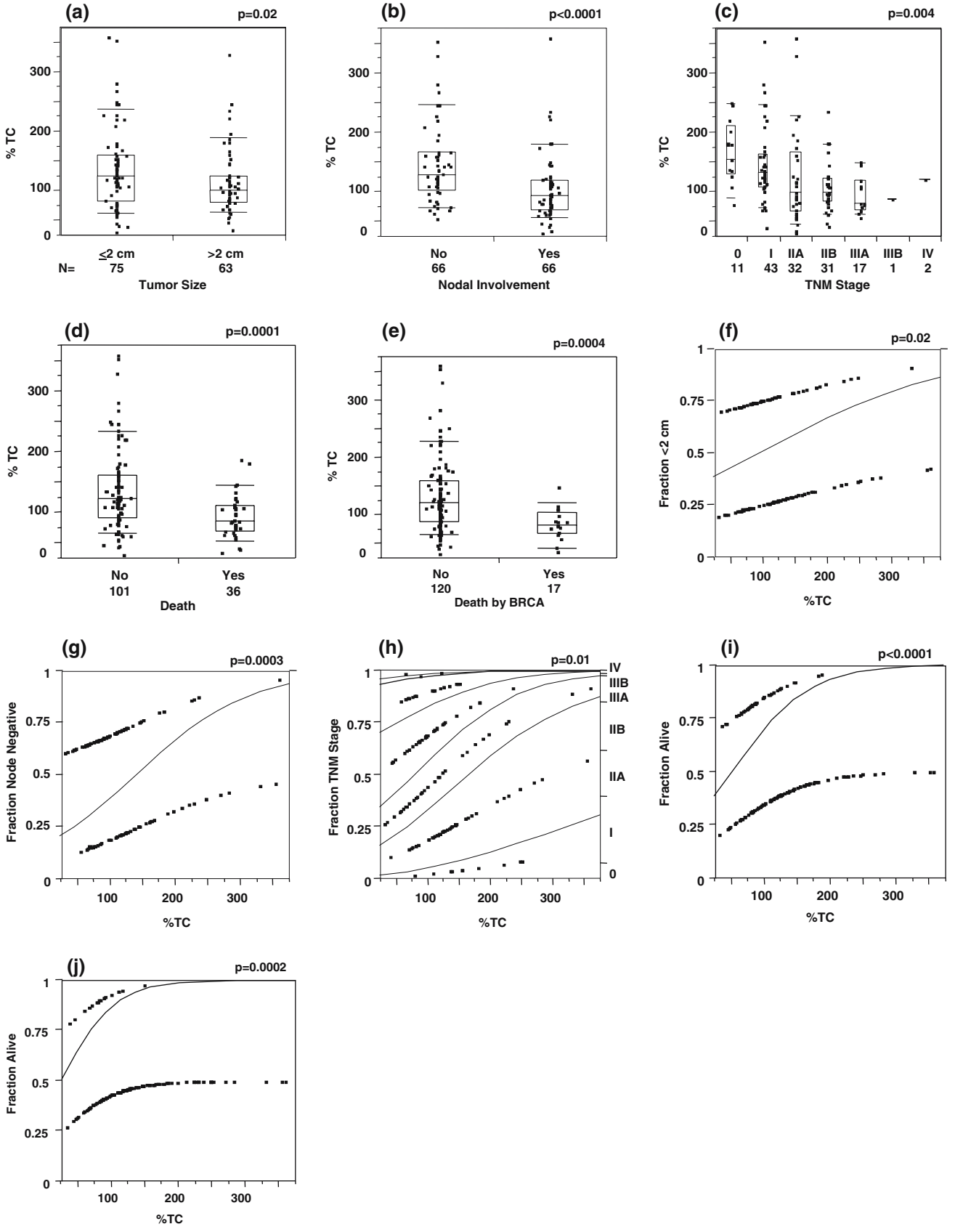
(a)
p=0.02
% TC
≤2 cm
>2 cm
N= 75 63
Tumor Size
(b)
p<0.0001
% TC
No
Yes
66 66
Nodal Involvement
(c)
p=0.004
% TC
0 I IIA IIB IIIA IIIB IV
11 43 32 31 17 1 2
TNM Stage
(d)
p=0.0001
% TC
No
Yes
101 36
Death
(e)
p=0.0004
% TC
No
Yes
120 17
Death by BRCA
(f)
p=0.02
Fraction <2 cm
%TC
(g)
p=0.0003
Fraction Node Negative
%TC
(h)
p=0.01
Fraction TNM Stage
IV
IIIB
IIIA
IIB
IIA
I
0
%TC
(i)
p<0.0001
Fraction Alive
%TC
(j)
p=0.0002
Fraction Alive
%TC

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

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

The sensitivity and specificity of TC as a predictor of breast cancer-related death was evaluated by analysis of the TC's receiver operating characteristics (Figure 3). TC ranges for the lower, middle and upper tertiles in normal tissues were <101, 101–123, and >123% of standard, respectively. Consistent with the data in Figure 1 demonstrating that many tumors have TC values that are greater or lesser than those typically observed in normal tissues, only 20 and 14% of tumors in the 1982–1993 and 1996–1999 sets, respectively, had TC values within the range defined by the middle tertile. The 124% cutoff predicted 5 year survival with approximately 50% sensitivity and 95% specificity, while the 100% TC cutoff predicted death due to breast cancer with approximately 75% sensitivity and 70% specificity.

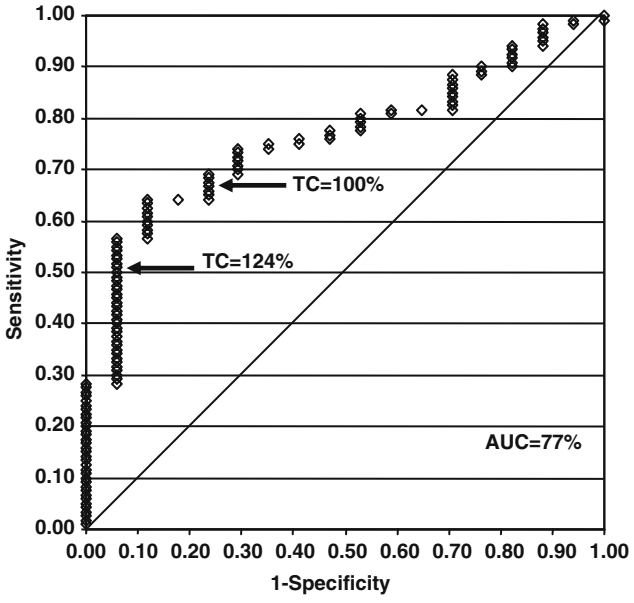

Figure 3. Receiver operating characteristics (ROC) analysis of the relationship between TC in breast tumors and 5 year survival. The specificity and sensitivity of TC as a predictor of 5 year survival following diagnosis of breast cancer was calculated for each value of TC in the combined 1982–1993 and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

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

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

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

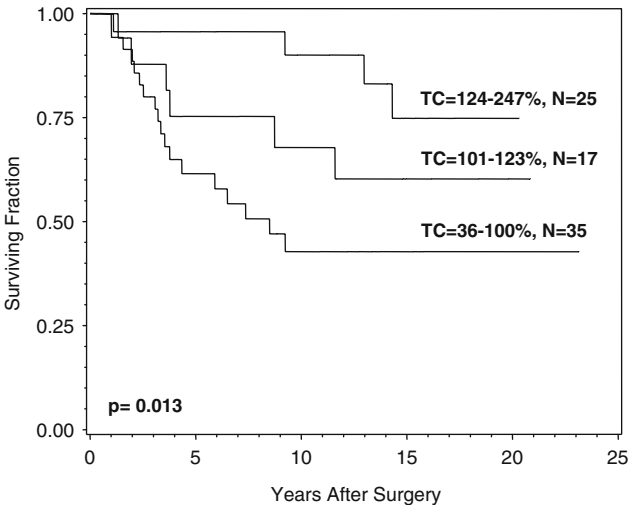

Figure 4. Breast cancer death by telomere DNA content in breast tumors. The 1982–1993 set was divided into three groups based on the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. Breast cancer-free survival interval, in years,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

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

	TC level					
	36–100%		101–123%		124–247%	
	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

Abbreviations: TC, telomere DNA content; *N*, number of specimens.
[a]Size is measured in longest dimension.

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

	Unadjusted		Adjusted for age, TNM stage	
	RH (95% CI)	*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.

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

Discussion

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

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

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

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

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

It is important to point out that all of the analyses reported herein were performed with DNA purified from tumor tissues that had *not* been microdissected. Although histological review of tissue sections indicated that tumor cells typically comprised 75–100% of the samples, the potentially confounding effects of contaminating normal cells in the tumor warrants consideration. In this context, we recently demonstrated that telomere attrition *comparable to that in matched prostate and breast tumor tissues* occurs in histologically normal tissues at distances at least one centimeter from the visible tumor margins [20,31]. In the latter study, it was estimated that at least 40% of the cells in the tumor adjacent histologically normal (TAHN) breast tissues were genetically aberrant, and more than a third of unbalanced alleles in the tumor were conserved in matched TAHN breast tissues, implying that the tumor and TAHN cells were derived from the same progenitor. Taken together, these data support the conclusion that TC in tumors and ''contaminating'' normal cells are comparable, thus precluding the requirement for tissue microdissection.

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

Acknowledgments

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