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A prospective study of relative telomere length and postmenopausal breast cancer risk

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Abstract

During breast cancer progression, a substantial increase in chromosomal aberrations is observed in the transition from ductal hyperplasia to carcinoma *in situ* (DCIS). Telomeres are essential structures to chromosomal integrity. Consequently, telomere dysfunction, which leads to genomic instability, is hypothesized to play a causal role in the progression of breast cancer. However, the few epidemiologic studies that have assessed the relationship between telomere length and breast cancer risk have been inconsistent. We used quantitative real-time PCR to measure relative telomere length in genomic DNA extracted from peripheral blood leukocyles and examined its association with postmenopausal breast cancer risk in 1122 invasive breast cancer cases and 1147 matched controls free of diagnosed cancer nested within the prospective Nurses' Health Study. Our data show that relative telomere length was not associated with a significant elevation in postmenopausal breast cancer risk (below vs. above median; OR = 1.23, 95% CI = 0.94–1.60, P_{trend} = 0.20). Estrone and estradiol hormone levels were significantly inversely associated with relative telomere length (p = 0.02). Other established breast cancer risk factors such as family history of breast cancer and history of benign breast disease were not associated with relative telomere length in separate linear regression models each adjusted for age and disease status ($p \geq 0.07$). Our results provide little support for an important role of telomere length, as measured in peripheral blood leukocyles, as a biomarker of breast cancer risk.

Keywords

telomere length; breast cancer; peripheral blood leukocyles

Introduction

Telomeres are long hexameric $(TTAGGG)_n$ repeats located at the distal ends of linear eukaryotic chromosomes. Recent studies have demonstrated the critical role of telomeres in maintaining the structural integrity of chromosomes by preventing nucleolytic decay, chromosomal end-to-end fusion, and atypical recombination (1). Due to limitations in lagging strand DNA synthesis at chromosomal ends, human telomeres shorten by ~50–100bp per mitotic division (2). Germline tissues express telomerase, which appends chromosome ends

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with hexameric repeats to restore telomere length. Most adult somatic tissues do not express telomerase resulting in the progressive loss of telomeric DNA with age. Therefore, telomeres are analogous to a "molecular clock" reflecting the number of divisions a cell has undergone (3).

When telomeres shorten to a critical length, a cell cycle checkpoint is triggered, proliferation is blocked, and the cell enters replicative senescence. However, if the Rb and p53 signalling pathways have been inactivated, cell division continues, resulting in further telomere shortening with a concurrent increase in genomic instability. Eventually, the dividing cell reaches crisis, a second proliferation block characterized by gross chromosomal aberrations. The vast majority of these cells undergo apoptosis. However, a rare cell may escape through the reactivation of telomerase, which at this stage is thought to facilitate tumor initiation and progression. Reactivation of telomerase is detected in >90% of human tumors, making it one of the most common abnormalities in cancer cells (1). Progressively shorter telomeres, as measured in peripheral blood leukocyles, have been associated with increased risk of head and neck, lung, renal cell, and bladder cancers (4–6) suggesting telomere length may be a useful biomarker of cancer risk.

An estimated 1.3 million women worldwide were diagnosed with breast cancer in 2007, making it the most common cancer in women and age is the most important risk factor (7). Patterns of telomere length, telomerase activity, genomic instability, and chromosomal aberrations observed throughout progressive stages of breast cancer implicate telomere crisis as a crucial tumor promoting event. This most likely occurs during the transition from usual ductal hyperplasia to carcinoma *in situ* (DCIS) (8). Few studies have investigated the association of telomere length with breast cancer risk and have found inconsistent results. A case-control study of high-risk sister sets (n = 287 cases) found an elevation in breast cancer risk predominantly among premenopausal women with short telomeres (9), whereas another study (n = 265 cases) found a significant increased breast cancer risk and decreased survival among a mix of pre- and postmenopausal women with longer telomeres (10). A third study found no association among participants from either the Twins UK registry (n = 72 treated cases) or a breast cancer screening population (n = 140 untreated cases) (11). To contribute to this unresolved question, we investigated the association between relative telomere length (RTL) and postmenopausal breast cancer risk in this study of 1122 cases and 1147 matched controls nested within the Nurses' Health Study.

Materials and Methods

Study population

The Nurses' Health Study is a prospective cohort study of 121,700 female registered nurses in 11 states in the United States who were 30–55 years of age at enrollment. In 1976 and biennially thereafter, self-administered questionnaires were used to gather detailed information on lifestyle factors, menstrual and reproductive factors, and medical history. During 1989–90, blood samples were collected from 32,826 women forming a subcohort from which cases and controls were selected. Eligible cases consisted of postmenopausal women with pathologically confirmed incident invasive breast cancer diagnosed anytime after blood collection up to June 1, 2004 with no prior diagnosis of cancer. Controls were randomly selected postmenopausal women free of cancer up to and including the questionnaire cycle in which the case was diagnosed. Controls were matched to cases according to age at diagnosis, blood collection variables [time of day, season, and year of blood collection, as well as recent (<3 months) use of postmenopausal hormones], and ethnicity (all cases and controls are self-reported Caucasians). Completion of the self-administered questionnaire and submission of the blood sample was considered to imply informed consent. The study protocol was approved by the Committee on Use of Human Subjects of the Brigham and Women's Hospital, Boston, MA.

Exposure data

Information on age at menarche, height, and age at first birth were obtained in 1976. Parity was collected biennially from 1976 to 1984. Weight at age 18 was queried in 1980. A history of breast cancer in a mother and/or sister was assessed in 1976, 1982, and every 4 years since 1988. Menopausal status, age at menopause, PMH use, weight, smoking information and diagnosis of benign breast disease were assessed at baseline and biennially thereafter. For each questionnaire women were asked whether their menstrual periods have ceased permanently, at what age and for what reason (natural or surgical). Participants were defined as postmenopausal if they reported having a natural menopause or bilateral oophorectomy. Women who reported a hysterectomy with either one or both ovaries remaining were defined as postmenopausal when they were 56 years old (if a nonsmoker) or 54 years old (if a current smoker), ages at which natural menopause had occurred in 90% of the respective cohorts. Age at menopause in the NHS is reported with a high degree of reproducibility and accuracy (12). Height and weight were used to calculate body mass index (BMI, kg/m^2). Number of cigarettes per day was reported in categories of usage (1–4, 5–14, 15–24, 25–34, 35–44, and 45 or more cigarettes per day). Smoking duration in years multiplied by packs of cigarettes smoked per day (20 cigarettes per pack) was used to calculate pack-years of smoking. All time-varying covariates were assessed in the questionnaire cycle prior to blood collection.

Relative telomere length (RTL)

Genomic DNA was extracted from peripheral blood leukocyles using the QIAmp (Qiagen, Chatsworth, CA) 96-spin blood protocol. PicoGreen DNA quantitation was performed using a Molecular Devices 96-well spectrophotometer. Genomic DNA was subsequently dried down and resuspended to ensure accurate and uniform DNA concentrations. The ratio of telomere repeat copy number to a single gene copy number (T/S) was determined by a previously described modified version (6) of the quantitative real-time PCR telomere assay (13). This PCR-based assay utilizes a high-throughput 384-well format of the Applied Biosystems 7900HT PCR System (Foster City, CA). Briefly, 5 ng of peripheral blood leukocyte derived genomic DNA was dried down in a 384-well plate and resuspended in 10 μL of either the telomere or 36B4 (single copy gene) PCR reaction mixture. Triplicate reactions of each assay were performed on each sample. RTL is reported as the exponentiated T/S ratio. Coefficients of variation (CV) of the telomere and single-gene assay were 1.03% and 0.56%, respectively. The CV for the exponentiated T/S ratio of quality control samples was 16.3%. After excluding extreme (2 cases, 1 control) and missing RTL (24 cases, 13 controls) values, our nested case-control study consisted of 1122 postmenopausal invasive breast cancer cases and 1147 matched controls.

Hormone measurements

Hormone assays were conducted from 1992 to 2003 in up to 6 batches. Assay methods have been described in detail elsewhere (14,15). Hormone levels were available on 499 women for estrone (250 cases, 249 controls), 636 women for estradiol (315 cases, 321 controls), and 624 women for estrone sulfate (311 cases, 313 controls).

Statistical analyses

We used a T-test to compare differences in continuous variables by disease status and a χ^2 test to compare the proportion of a first-degree family history of breast cancer between the groups. The Wilcoxon signed rank test was used to analyze RTL by disease status. In subsequent analyses, we used the control-specific distribution to categorize RTL by median or quartile values and continuous RTL was natural logarithm transformed to satisfy the assumption of normality. Linear regression was used to examine age-adjusted associations between RTL and risk factors among cases and controls separately, then among all subjects controlling for disease

status. We used the Wald test to test for additive interactions between disease status and risk factors listed in Table 2. We used generalized linear models to regress the natural logarithm of estrone, estradiol, or estrone sulfate on RTL quartiles, adjusted for age, disease status, laboratory batch, and matching factors. Multivariate-adjusted associations between RTL quartiles and postmenopausal breast cancer risk were examined using conditional logistic regression to calculate odds ratios (OR) and 95% confidence intervals (CI). Covariates included in the multivariate models are listed in Table 3. The *P* values are two-sided; *P* values < 0.05 were considered statistically significant. We used the SAS Version 9.1 software (SAS Institute, Cary, NC).

Results

Our analyses included 1122 postmenopausal breast cancer cases and 1147 age-matched controls. The mean ± SD age of diagnosis for women with breast cancer was 65.7 ± 6.7 years (range, 44–83). Age at blood draw and age at diagnosis were similar among cases and controls as expected from the matched design. On average, women with breast cancer had greater pack-years of smoking and were statistically significantly more likely to have a family history of breast cancer among first-degree relatives ($p < 0.01$). Cases and controls had similar average relative telomere length (RTL) and BMI (Table 1).

We examined the relationship of RTL with factors hypothesized to influence telomere length (i.e., age, smoking, postmenopausal hormone use, and BMI) and with established breast cancer risk factors (Table 2). Of the factors hypothesized to affect telomere length, age showed a statistically significant inverse relationship with RTL. No significant associations were observed between RTL and smoking, duration of postmenopausal hormone use, or BMI. Statistically significant inverse associations were observed between RTL and plasma estrone ($p = 0.02$) and estradiol ($p = 0.02$) among subjects with measured hormone levels. Weight gain since age 18, age at menarche, age at first birth, parity, age at menopause, family history of breast cancer, and personal history of benign breast disease were not associated with RTL. Some evidence of heterogeneity by disease status was observed for parity ($p = 0.05$), with a suggestive inverse association with RTL among cases ($p = 0.08$), but not controls ($p = 0.33$).

We also investigated the relationship between RTL and postmenopausal breast cancer risk (Table 3). We did not observe a significant increase in breast cancer risk among women with shorter RTL. After adjustment for known breast cancer risk factors, women with an RTL below the median were at a non-significant elevated risk of postmenopausal breast cancer compared to women with an RTL above the median (OR = 1.23, 95% CI = 0.94–1.60). With increasing quartiles of RTL, a non-significant inverse trend was observed with postmenopausal breast cancer risk ($P_{trend} = 0.20$). Results were similar upon division of RTL into deciles (data not shown). No statistically significant associations or trends were observed between RTL and breast cancer risk when analyses were stratified by family history of breast cancer or ER/PR status (data not shown).

Discussion

We examined whether excessive telomere shortening may lead to breast cancer by investigating the relationship between relative telomere length (RTL) and breast cancer risk in a large breast cancer case-control study nested within the prospective Nurses' Health Study. We found a non-significant 25% elevation in postmenopausal breast cancer risk among women in the shortest quartile of RTL compared to women in the longest quartile. This is somewhat comparable to a case-control study of high-risk sister sets, which reported a non-significant 34% increased risk of postmenopausal breast cancer among women in the shortest versus longest quartile of relative telomere. However, the authors did not observe a consistent trend. Instead the

association of telomere length with breast cancer risk, though still not statistically significant, appeared predominantly in premenopausal women (9). All women in our study were postmenopausal at diagnosis, limiting the generalizability of our results to postmenopausal women.

Telomere length did not differ between 123 untreated newly diagnosed breast cancer cases and 108 age- and ethnicity-matched controls in a small UK study (11), whereas a Swedish case-control study observed unexpected associations with increased breast cancer risk and decreased survival associated with longer RTL (10). The potential for systematic measurement bias of RTL is of some concern in the Swedish study as DNA was isolated from granulocytes in 1/3 of control subjects, while buffy coat DNA was isolated from cases and the remainder of controls. Within the same individual, granulocytes may have telomeres as much as ~2–3 kb shorter than leukocyte telomeres up until age 60 (16). As the majority of controls in the Swedish study were less than 60 years of age, the inclusion of short telomere measurements from granulocytes may have created a spurious positive association between RTL and breast cancer risk.

We also assessed the relationship between RTL and several known breast cancer risk factors. Other than the statistically significant inverse association with age at blood draw ($P_{trend} < 0.01$), most of the established breast cancer risk factors were not significantly associated with RTL. Inverse associations between RTL and estradiol and estrone hormone levels reached statistical significance. These associations appeared stronger in cases than in controls, though no significant heterogeneity was observed by disease status ($p \geq 0.23$). When cases diagnosed within the first 2 years of follow-up were excluded, we no longer observed inverse trends with RTL (data not shown) suggesting a potential effect of underlying disease. Greater local concentration of estrogens in cancerous versus normal breast tissue (17) could have subtly increased peripheral plasma concentrations resulting in the observed associations. One study demonstrated that estradiol upregulates telomerase expression *in vitro* (18), but how this translates to the *in vivo* relationship between telomere length and plasma hormone levels among postmenopausal women has not been explored.

Besides being the largest study to date, our analyses benefit from the nested case-control design. In addition to drawing cases and controls from a well-characterized relatively homogeneous population limiting selection bias, the collection of blood specimens occurred prior to breast cancer diagnosis. This reduces the potential of generating invalid risk estimates due to the limitations of retrospective case-control studies, such as the cancer treatment and/or the disease itself influencing the phenotype of interest, i.e. telomere length. Furthermore, given that telomere length has been positively associated with survival (19), cancer patients with shorter telomere lengths may die or be too ill to participate in a case-control study resulting in a case group with a disproportionate number of individuals with longer telomeres. The number of deaths attributed to breast cancer (n = 29) was not sufficient to investigate the relationship between RTL and breast cancer survival.

We used an economical real time PCR-based method in a high-throughput setting to measure RTL in DNA derived from peripheral blood leukocyles. While this does not provide an absolute measurement of telomere length, relative telomere lengths estimated by this method correlate well with telomere restriction fragment (TRF) lengths produced by the Southern blot assay (13). An advantage of the PCR-based assay over the TRF assay is that it does not measure subtelomeric DNA, which can introduce up to 2 kb in variation between individuals (13). In our study, we observed a statistically significant inverse correlation with age giving further assurance that the real-time PCR method provides a biologically meaningful measure of telomere length. We cannot be certain that RTL measured in blood reflects telomere length in breast tissue. However, statistically significant correlations found between leukocyte telomere

length and other tissues from the same individual suggest blood serves as an adequate proxy for non-malignant breast tissue (20).

In summary, we did not observe a significant elevation in postmenopausal breast cancer risk associated with shorter telomere lengths. Our data provide little support for an important role of telomere crisis as a crucial factor in breast carcinogenesis among postmenopausal women. Additional prospective studies are needed to confirm our finding as well as to explore the relationship between relative telomere length, premenopausal breast cancer risk, and breast cancer survival.

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Table 1

Select population characteristics of breast cancer cases and controls from the Nurses Health Study

Variable	Cases, n=1122	Controls, n=1147	P-value
Age at blood draw*, mean (SD)	58.4 (6.5)	58.4 (6.4)	0.98
Age at diagnosis*, mean (SD)	65.7 (6.7)	65.6 (6.7)	0.78
Relative telomere length, mean (SD)	17.8 (6.6)	17.9 (7.0)	0.90
Relative telomere length†, median	16.7	16.9	0.64
Pack-years of smoking at blood draw‡, mean (SD)	24.0 (20.9)	21.9 (18.1)	0.07
BMI at blood draw, mean (SD)	25.5 (4.6)	25.4 (4.6)	0.58
Family history of breast cancer§ (%)	24.2	17.7	<0.01

*Age was a matching factor.

†P-value obtained using the Wilcoxon signed rank test on 1105 case-control pairs.

‡Among cigarette smokers only.

§P-value obtained by chi-square test.

Table 2

Age-standardized characteristics by relative telomere length quartiles among all subjects

Characteristic	Quartile 1	Quartile 2	Quartile 3	Quartile 4	p-values
Age (mean)	59.2	58.7	58.0	57.7	<0.01
Packyears (ever smokers)	24.3	22.2	22.0	23.4	0.27
Packyears (past smokers)	19.3	16.2	16.9	17.0	0.09
Cigarettes/day (ever smokers)*	2.78	2.78	2.72	2.67	0.21
Cigarettes/day (past smokers)*	2.78	2.71	2.64	2.59	0.12
PMH duration (years)	4.28	3.63	4.03	3.51	0.07
BMI (kg/m^2)	25.3	25.6	25.3	25.5	0.33
Weight gain since 18 (kg)	11.3	11.4	11.2	11.5	0.49
Age at menarche	12.5	12.5	12.6	12.6	0.57
Age at first birth	25.1	25.4	25.3	25.3	0.40
Parity	3.10	2.99	3.02	3.02	0.59
Age at menopause	49.7	49.9	49.6	49.6	0.37
Family history of breast cancer (%)	20	18	23	22	0.17
History of benign breast disease (%)	60	58	58	58	0.85
Estrone (pg/ml)	27.4	27.7	27.1	24.3	0.02
Estradiol (pg/ml)	7.47	7.75	6.72	6.76	0.02
Estrone Sulfate (pg/ml)	241	237	232	210	0.09

*Cigarettes/day were recorded in categories of 1:1–4, 2:5–14, 3:15–24, 4:25–34, 5:35–44, 6:≥45 cigarettes/day.

Table 3

Association between relative telomere length and breast cancer risk

Relative telomere length	Cases, n (%)	Controls, n (%)	OR (95% CI)*	OR (95% CI)†
4th quartile	221 (24.7)	223 (24.3)	1.00	1.00
3rd quartile	209 (23.3)	228 (24.9)	0.95 (0.70 – 1.28)	0.95 (0.69 – 1.30)
2nd quartile	243 (27.1)	235 (25.6)	1.14 (0.81 – 1.59)	1.16 (0.81 – 1.65)
1st quartile	223 (24.9)	231 (25.2)	1.17 (0.80 – 1.73)	1.25 (0.83 – 1.88)
			P_{trend} = 0.32	P_{trend} = 0.20
Above median	430 (48.0)	451 (49.2)	1.00	1.00
Below median	466 (52.0)	466 (50.8)	1.19 (0.93 – 1.53)	1.23 (0.94 – 1.60)

*Odds ratios and 95% confidence intervals from conditional logistic regression analysis adjusted for matching factors (age, fasting status, recent postmenopausal hormone use, date and time of blood draw).

†Odds ratios and 95% confidence intervals from conditional logistic regression additionally adjusted for smoking status at blood draw, age at menarche, BMI at blood draw, weight gain from age 18 until blood draw, age at first birth and parity, family history of breast cancer, history of benign breast disease, age at menopause, duration of postmenopausal hormone use.