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Telomere length in peripheral blood and breast cancer risk in a prospective case-cohort analysis: results from the Sister Study

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Abstract

Objective—Telomeres are required for maintaining genomic integrity and may play a role in carcinogenesis. Some, but not all, epidemiologic studies have found that short telomeres in leukocytes are associated with an increased risk of breast cancer. To further elucidate this potential association, we examined telomere length in relation to breast cancer risk in prospectively collected blood samples from the Sister Study, a cohort of women aged 35–74 years who have a sister with breast cancer.

Methods—We performed a case-cohort analysis comparing incident breast cancer cases (n=342) with a subcohort (n=735), randomly selected from 29,026 participants enrolled by June 1, 2007. Relative telomere length in peripheral blood cells was estimated using a single tube monochrome multiplex quantitative PCR assay.

Results—No association was observed between telomere length and breast cancer risk. Compared to the longest quartile, hazard ratios (HR) associated with the second, third and the shortest quartile were 0.91 (95% confidence interval [95% CI]: 0.62–1.34), 1.11 (95% CI: 0.77–1.60) and 0.93 (95% CI: 0.64–1.35), respectively. Subgroup analyses by menopausal status, invasiveness or estrogen-receptor status of breast cancer did not reveal evidence of association between telomere length in blood cells and subsequent breast cancer risk.

Conclusions—This prospective investigation does not support telomere length in blood cells as a biomarker for breast cancer risk.

Keywords

breast cancer; telomere length; prospective study; biomarker; qPCR

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Introduction

Telomeres are non-coding double-stranded repeats of G-rich tandem sequences (TTAGGG in humans) located at ends of chromosomes that play a critical role in maintaining genomic integrity [1]. Experimental studies indicate that telomere dysfunction may be an important cause of chromosomal abnormalities in breast epithelium [2]. Chromosomal instability and burden of epithelial tumors both markedly increase in p53-deficient mice with telomere dysfunction [3,4], and comparative analyses of breast tissues using direct telomere fluorescence *in situ* hybridization technique have demonstrated progressively shorter telomere length with increasing chromosomal aberrations in breast carcinomas (from hyperplasia to carcinoma *in situ* and invasive cancer) compared to normal ductal epithelium [5,6]. However, epidemiologic studies examining the association between leukocyte telomere length and breast cancer have yielded equivocal results. Short telomeres have been reported to be associated with both an increased risk of breast cancer [7,8], particularly in women younger than 50 years [9], and a reduced risk [10,11]. More recent studies have shown no significant associations between telomere length and breast cancer [8,12,13]. To further elucidate the potential association, we examined telomere length in peripheral blood in relation to breast cancer risk in a prospective cohort study of women who have at least one sister with breast cancer.

Materials and Methods

Study design

The Sister Study is a prospective cohort study to investigate environmental and genetic risk factors for breast cancer and other end points in 50,884 women aged 35-74 years. To be eligible, women cannot have had breast cancer at time of enrollment but are at increased risk by virtue of having a sister(s) with breast cancer [14]. Eligibility was not based on carrier status for BRCA1 or BRCA2 mutations. At baseline, study participants provided information on various potential risk factors such as reproductive history, medication use and family history of breast and other cancers, and provided a blood specimen during a home visit.

To examine the association between relative telomere length in blood and breast cancer risk, we performed a case-cohort analysis in which incident breast cancer cases (n=342) were compared with a subcohort (n=735), randomly selected from 29,026 participants who had completed the baseline questionnaire and home visit by June 1, 2007. Participants reported breast cancer diagnoses on annual and biennial health questionnaires, or by calling the Sister Study helpline. One year after the self-reported diagnosis date, women were contacted for information regarding their diagnosis and treatment and asked to authorize release of pertinent medical records. By the time of the present analysis, the diagnosis was confirmed by pathology reports or medical records in 76% of cases (n=260). However, our analysis did not exclude self-reported cases whose pathology reports were not yet obtained because the accuracy of self-reporting was high (98%). Similarly, there was good agreement between self-report and medical records for estrogen receptor status (95%) and invasiveness (81%). Therefore, when medical records were not available, information from self-report was used in subgroup analyses by tumor characteristics. Still, some cases lacked information on tumor invasiveness (n=40) and estrogen receptor status (N=48), and were excluded from corresponding analyses. The study was approved by the Institutional Review Board of the National Institute of Environmental Health Sciences, NIH and the Copernicus Group Institutional Review Board.

Laboratory methods

Genomic DNA was extracted from frozen blood samples on the Qiagen Autopure LS in the NIEHS Molecular Genetics core facility. Extracted DNA was eluted in TE buffer and stored at -20°C following quantification using the Quant-iT™ PicoGreen dsDNA reagent (Invitrogen). 10 ng aliquots of DNA were robotically plated in duplicate onto each of 4 replicate 384-well plates.

Telomere length was determined as the relative ratio of telomere repeat copy number to single copy gene copy number (T/S ratio) using the monochrome multiplex quantitative PCR protocol described by Cawthon [15]. Telomere primer sequences were telg (ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT), telc (TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA), and albumin was used as the single copy gene reference using primers modified with the addition of a 5'-GC clamp to shift melting temperature: albu (CGGCGGCGGGCGGCGGGCTGGGCGGaaatgctgcacagaatccttg) and albd (GCCCCGCCCCGCGCGCCCGTCCCGCCGaaaagcatggtcgctgtt). The reagent components and final concentrations were 900 nM each primer (IDT), 1X AmpliTaq Buffer II (ABI), 3 mM MgCl₂, 0.2 mM per dNTP, 1 mM DTT, 1 M betaine, 0.75X SYBR Green I and 0.625 U AmpliTaq Gold polymerase.

A 5-point standard curve was included in quadruplicate on each assay plate. DNA for the standard curve was made from a pool of normal subjects and ranged from 1.9 to 75 ng in a 2.5-fold dilution series run. Each plate also contained quadruplicate samples of three controls selected for high, medium and low T/S ratio values. Plates were run on a BioRad CFX384 (Hercules, CA) with the following cycling parameters: 95°C for 15 minutes; 2 cycles at 94°C for 15 seconds, 49°C for 15 seconds; 33 cycles at 94°C for 15 seconds, 62°C for 10 seconds, 74°C for 15 seconds, 84°C for 10 seconds, 88°C for 15 seconds. Signal acquisition at 74°C allowed for collection of the telomere Ct values, while acquisition at 88°C provided the albumin Ct values. Biorad CFX Manager software automatically estimated the value for each sample T (telomere) and S (albumin single copy) using the standard curve. Standard curve efficiencies for both primer sets were above 90%, and regression coefficients were at least 0.99 in all PCR runs. Plates were verified for overall quality control parameters. All study samples were run in duplicate wells over four different PCR plates. Average coefficient of variation (%CV) was 11% and intraclass correlation coefficient (ICC) of a single T/S ratio was 0.85. Individual telomere length was obtained from the average of up to eight replicate T/S ratio values.

Statistical analyses

Hazard ratios (HR) and 95% confidence intervals (95% CI) for the association between telomere length and breast cancer were estimated based on a case-cohort analysis using Prentice's pseudo-likelihood with modification of the standard errors based on robust variance estimates [16]. The pseudo-likelihood is a weighted Cox regression model, and different weighting methods have been proposed [17]. In our analysis, choice of weighting method did not affect the estimates; therefore, we report estimates obtained using Barlow's weighting scheme where case weight is one but subcohort members are weighted by the inverse of the sampling fraction [17]. The primary time scale used was age time, and women were delay-entered into risk sets (left-censored) beginning at the age when they completed enrollment with follow-up continuing until their age at diagnosis of breast cancer for cases and the earlier of final non-response date or the follow-up cutoff date of May 15, 2008 in non-cases.

We examined the following potential confounders: race (white or non-whites), education (high school, some college, associate or technical degree, college degree, or post college), mother and sister's history of breast cancer (sister diagnosed aged ≥ 50 years sister diagnosed aged < 50 years, mother diagnosed at any age and sister diagnosed aged ≥ 50 years, mother diagnosed at any age and sister diagnosed aged < 50 years), body mass index (< 25 , 25-29.9, ≥ 30 kg/m²), alcohol use (never, past drinker with < 10 drinks/year, current drinker with < 10 drinks/year, past drinker with ≥ 10 drinks/year, or current drinker with ≥ 10 drinks/year), smoking status (never, past, or current), age at menarche (< 12 , 12-14, 15-19 years), first pregnancy at < 30 years of age, menopause status at the time of enrollment, and years of using hormone replacement therapy (< 3 , 3-9, 10-19, ≥ 20 years). However, adjustment for these factors did not change estimates or significance levels, and therefore, we report only the unadjusted estimates. Subgroup analyses were done by treating tumor occurrences in the subcohort that were assigned to types not under consideration as censoring events. Tests for linear trend were done by treating an ordered categorical variable as a continuous variable. Significance tests were two-sided with the level of significance at 0.05. Stata 10.0 (College Station, TX) was used for all the analyses.

Results

Baseline characteristics of the subcohort members by quartiles of relative telomere length are presented in Table 1. Telomere length was weakly inversely associated with age. Women with shorter telomeres were slightly more likely to have their first pregnancy before 30 years of age. They were also more likely to be postmenopausal and to have used hormone replacement therapy compared to those with longer relative telomere length, but those associations became nonsignificant with adjustment for age. Other factors such as obesity, current smoking/drinking, physical activity and mother's history of breast cancer did not display any consistent pattern associated with telomere length, regardless of age-adjustment.

Average telomere length was similar in cases and subcohort members with mean of 1.25 (SD=0.37) in the cases and 1.24 (SD=0.35) in subcohort. Eleven women in the subcohort subsequently developed breast cancer; mean telomere length in the subcohort remained the same after excluding these cases. There was no association between relative telomere length and breast cancer risk (Table 2). Compared to the longest quartile of telomere length, HRs associated with short telomeres were 0.91 (95% CI: 0.62-1.34) for the second quartile, 1.11 (95% CI: 0.77-1.60) for the third quartile and 0.93 (95% CI: 0.64-1.35) for the shortest quartile, respectively. We also performed subgroup analyses by menopause status, obesity, age at enrollment, time between enrollment and diagnosis, and tumor invasiveness or estrogen receptor status. However, there was no evidence of effect modification by any of these subanalyses or etiologic heterogeneity across tumor characteristics.

Discussion

In this prospective cohort of women aged 35-74 years, no association was observed between relative telomere length in blood and breast cancer risk. Short telomeres were rather associated with a decreased risk of breast cancer in several subgroups; however, these associations were not significant and showed no indication of linear trend. Our finding of no association between telomere length and breast cancer risk is consistent with recent reports including two prospective investigations [8,12,13]. On the other hand, short telomeres were associated with either an increased [9,18] or a decreased [10,11] risk of breast cancer in case-control studies where telomere length was quantified in blood after diagnosis of cancer. This could reflect possible reverse causation in case-control studies. It has been reported that individual patients underwent various changes in leukocyte telomere length after completion of adjuvant standard-dose chemotherapy commonly used for breast cancer [19]. The present

prospective study is not subject to potential bias due to reverse causality by treatment effect. Although the follow-up time was relatively short with mean of 460 days in cases in this study, the stratified analysis by time between blood draw and diagnosis showed no difference in the association with telomere length by recency of diagnosis suggesting that telomere length in blood is neither indicator of future breast cancer risk nor a marker for the presence of breast cancer prior to clinical diagnosis.

The present study introduced several innovations aimed at enhancing both accuracy and precision in telomere length measurement. First, we determined individual telomere length in up to 8 technical replicates run across 4 PCR plates in all study samples. We also adopted a single-tube monochrome multiplexing PCR method, which is an improved alternative to the conventional singleplex (two tube) PCR-based method [15] because the T/S ratio is no longer affected by the pipetting-induced variation in DNA amount[15]. With this method, however, %CV in our study was not particularly improved from some of the previously reported [11,12,20]. This might be partly explained by different methods calculating %CV found in the literature including computation of %CV based on variability of threshold cycle values [20], or computation using only the remaining measurements after excluding outliers, with outliers being defined in various manners [11,12]. Such approaches would have reduced our reported %CV. For example, we performed sensitivity analyses recalculating individual's average telomere length after excluding technical outliers based on several predefined criteria. While we could reduce %CV as low as 5% under different definition of outlier, we observed that there were very few technical outliers regardless of the methods chosen, and exclusion of the few outliers did not impact the observed association between telomere length and breast cancer (data not shown). Since we found no influence of outlier on the association of interest, we used all the measurements in our analysis and reported conservative %CV based on T/S ratio.

In any studies, a null finding could have been caused by insufficient power. However, our current sample size was previously estimated to be sufficient to detect a significant difference of 0.08 (assuming $SD=0.4$) in mean relative telomere length between cases and non-cases. The mean difference in telomere length of 0.01($SD=0.37$) observed in the present study might have become statistically significant with much bigger sample size, but it is doubtful that such small mean difference, which can be converted to approximately 42 base pairs according to a validation study [21], would have a biologic implication or any clinical relevance.

Accumulating evidence from experimental studies clearly supports the role of telomere regulation in the development and progression of cancer: unrestrained telomere shortening and activation of telomerase may lead to genomic instability that accelerates accumulation of genetic aberrations for cellular immortalization and cancer development [3]. Telomere length in blood, which may record a replicative history of hematopoietic stem cells and progenitor cells, has been associated with chronological age, obesity and smoking [22,23]. However, our data did not suggest that telomere length in blood is associated with telomere function in distant tissues like breast.

In conclusion, we used prospectively collected blood samples to conduct a case-cohort analysis of telomere length and breast cancer and did not find evidence supporting telomere length in blood as a biomarker for breast cancer risk. It is unlikely that telomere length in blood is an important predictor of breast cancer risk.

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Table 1

Baseline characteristics of subcohort members (n=736) across the quartiles of relative telomere length in the Sister Study

	<1.0	1.0-<1.18	1.18-<1.42	1.42		
No	182	183	183	187	p*	p [†]
<i>Mean(SD)</i>						
Age	57.7 (9.1)	54.4 (8.8)	53.2 (8.6)	52.6 (9.2)	<0.01	
N days of follow-up	889.0 (259.2)	878.5 (309.1)	879.0 (302.8)	948.8 (324.4)	0.07	0.07
Physical activity (MET-hours/week)	15.7 (23.3)	13.7 (14.1)	17.8 (21.3)	15.0 (15.0)	0.79	0.72
<i>No (%)</i>						
Non-white race	11 (6.0)	11 (6.0)	28 (15.3)	10 (5.4)	0.44	0.70
High school education	21 (11.5)	26 (14.2)	25 (13.7)	22 (11.8)	0.98	0.54
BMI 30 kg/m ²	50 (27.5)	41 (22.4)	51 (28.0)	49 (26.3)	0.88	0.96
Current alcohol drinks						
10/year	137 (75.3)	155 (84.7)	140 (76.5)	143 (76.5)	0.73	0.21
Current smokers	13 (7.1)	14 (7.7)	15 (8.2)	13 (7.0)	1.00	0.49
Postmenopause	127 (69.8)	117 (63.9)	113 (61.8)	101 (54.0)	<0.01	0.71
Ever use of HRT	105 (58.0)	91 (50.0)	89 (48.6)	82 (43.9)	<0.01	0.99
Age at first pregnancy <30 y	146 (80.7)	141 (77.1)	139 (76.0)	141 (75.4)	0.23	0.91
Age at menarche<12y	36 (19.8)	36 (19.7)	38 (20.8)	28 (15.0)	0.30	0.28
Having mother diagnosed with breast cancer	29 (16.6)	38 (21.4)	33 (18.8)	36 (19.9)	0.58	0.60

* P for linear trend

[†] P for linear trend after adjustment for age at baseline (35-39, 40-44, 45-49, 50-54, 55-59, 60-64, 65-69, and 70-75 years)

Table 2

Hazard ratio (HR) of breast cancer according to quartiles of relative telomere length

	Case	Subcohort	HR	95% CI	P*	Cases	Subcohort	HR	95% CI	P*
<u>Entire case-cohort</u>										
1.42	92	187	1.00		0.97					
1.18-<1.42	73	183	0.91	(0.62-1.34)						
1.0-<1.18	96	183	1.11	(0.77-1.60)						
<1.0	81	182	0.93	(0.64-1.35)						
<u>Premenopausal women</u>										
1.42	42	86	1.00		0.75	<u>Postmenopausal women</u>				
1.18-<1.42	23	70	0.81	(0.43-1.52)		50	101	1.00		0.97
1.0-<1.18	42	66	1.30	(0.73-2.31)		50	113	0.99	(0.59-1.65)	
<1.0	23	55	0.94	(0.50-1.78)		54	117	1.03	(0.63-1.69)	
						58	127	0.91	(0.56-1.46)	
<u>BMI < 27.5 kg/m²</u>										
1.42	55	107	1.00		0.59	<u>BMI 27.5 kg/m²</u>				
1.18-<1.42	40	107	0.79	(0.47-1.31)		37	79	1.00		0.96
1.0-<1.18	59	113	0.98	(0.61-1.57)		33	75	0.98	(0.52-1.83)	
<1.0	45	104	0.81	(0.49-1.34)		37	70	1.11	(0.60-2.03)	
						35	79	0.95	(0.52-1.73)	
<u>Age at baseline < 55 year</u>										
1.42	50	105	1.00		0.71	<u>Age at baseline ≥ 55 years</u>				
1.18-<1.42	32	92	0.82	(0.48-1.41)		42	82	1.00		0.78
1.0-<1.18	49	95	1.04	(0.63-1.73)		41	91	1.00	(0.57-1.75)	
<1.0	25	67	0.81	(0.45-1.44)		47	88	1.17	(0.69-1.99)	
						56	115	1.04	(0.63-1.72)	
<u>Follow-up only including first year after baseline</u>										
1.42	43	187	1.00		0.46	<u>Follow-up only beginning at 1 year after baseline</u>				
1.18-<1.42	31	183	0.76	(0.44-1.32)		49	183	1.00		0.80
1.0-<1.18	43	183	0.95	(0.57-1.60)		42	181	1.01	(0.62-1.66)	
<1.0	31	182	0.75	(0.44-1.28)		53	178	1.17	(0.73-1.87)	
						50	178	1.02	(0.63-1.63)	
<u>Invasive cancer only</u>										
1.42	61	187	1.00		0.86	<u>ER+ invasive cancer only</u>				
1.18-<1.42	44	183	0.86	(0.55-1.34)		51	187	1.00		0.84
						39	183	0.91	(0.57-1.47)	

	Case	Subcohort	HR	95% CI	P*	Cases	Subcohort	HR	95% CI	P*
1.0-<1.18	63	183	1.09	(0.72-1.67)		51	183	1.07	(0.68-1.68)	
<1.0	56	182	0.97	(0.63-1.50)		44	182	0.90	(0.56-1.45)	

* P for linear trend