Telomere deficiencies on chromosomes 9p, 15p, 15q and Xp: potential biomarkers for breast cancer risk

Yun-Ling Zheng[1,*], Xin Zhou[1], Christopher A. Loffredo[1], Peter G. Shields[1] and Bing Sun[2]

[1]Carcinogenesis, Biomarkers and Epidemiology Program, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA and [2]BioMarkers, Rockville, MD, USA

Although telomere dysfunction is a characteristic of breast cancer cells, the relationship between deficiency on individual chromosomal telomeres in normal somatic cells and breast cancer risk has not been characterized. A case–control study was conducted to examine the associations between individual lengths of 92 telomeres in the human genome and the risk of breast cancer in 204 newly diagnosed breast cancer patients and 236 healthy controls. Chromosome arm-specific telomere lengths were measured by telomere quantitative fluorescent *in situ* hybridization. Unconditional logistic regression was used to estimate the risk associations. This genome-wide screen identified that shorter telomere lengths on chromosomes Xp and 15p were associated with breast cancer risk in pre-menopausal women, with adjusted odds ratios (aORs) of 2.5 (95% CI = 1.3, 4.8) and 2.6 (1.3, 5.0), respectively. The study also revealed that greater length differences between homologous telomeres on chromosomes 9p, 15p and 15q were associated with breast cancer risk in pre-menopausal women, with aORs of 4.6 (2.3, 9.2), 3.1 (1.6, 6.0) and 2.8 (1.4, 5.4), respectively. When the subjects were categorized into quartiles, a dose–response relationship was observed for all of the above telomeres (P-for-trend $\leq$ 0.005). This study revealed that telomere deficiencies on chromosomes 9p, 15p, 15q and Xp were associated with breast cancer risk in pre-menopausal women. If confirmed in future studies, chromosomal arm-specific telomeres are likely to be a useful panel of blood-based biomarkers for breast cancer risk assessment, given their strong associations with breast cancer risk.

INTRODUCTION

Cancer genomes are highly rearranged and are characterized by complex translocations and regional copy number alterations (1). Efforts to uncover the underlying mechanisms driving chromosome instability in cancer have revealed a prominent role for telomeres (2). Telomeres, the nucleoprotein complexes at the end of eukaryotic chromosomes, are specialized structures that protect chromosome ends and prevent them from being recognized by the cell as DNA double-strand breaks (3). Telomeres are vulnerable due to progressive shortening during each round of DNA replication (4) and telomere length is directly related to the proliferative history of the cell. Thus, a lifetime of tissue renewal places the organism at risk for telomere dysfunction and increasing chromosomal

instability, particularly in aged populations. Dysfunctional telomeres result in inappropriate chromosomal end-to-end fusions through the non-homologous end-joining or homologous recombination (HR) DNA repair pathways (5). These fusions are the basis of chromosome instability through repetition of the breakage–fusion–bridge cycle, causing chromosome abnormalities that are typically observed in most human cancer cells (6,7).

Although there is compelling evidence that telomere dysfunction (very short or extremely long telomeres) and chromosome instability are characteristics of breast cancers (8–10), the relationship between telomeres and breast cancer risk has not been adequately characterized. Several case–control studies have examined overall telomere length in blood leukocytes and risk of breast cancer (11–16) and reported

*To whom correspondence should be addressed at: Carcinogenesis, Biomarkers and Epidemiology Program, Lombardi Comprehensive Cancer Center, Georgetown University, 3800 Reservoir Road, NW, Box 571465, Washington, DC 20057, USA. Tel: +1 2026876654; Fax: +1 2027843034; Email: yz37@georgetown.edu

inconsistent results, with the majority of the studies (11,12,14,15) reporting no significant association. One of the major limitations of these previous studies is that only the overall telomere length (average telomere length of 92 telomeres in the human genome) was measured. Telomere lengths on each chromosomal end were not assessed. To shed light on the potential roles of specific chromosomal telomeres in breast cancer susceptibility, we conducted the first genome-wide telomere association study to examine the associations between lengths of 92 telomeres in blood lymphocytes and breast cancer risk.

RESULTS

Characteristics of study population

Table 1 lists the characteristics of the study subjects. The mean age was 53.0 for cases and 53.2 for controls. There were no significant case–control differences in the distributions of race, menopausal status, tobacco smoking status, alcohol use, education levels, family history of cancer, levels of household income and hormone replacement therapy (HRT) use. The mean body mass index was similar between cases and controls. Controls were significantly more likely to be physically active in their teenage years compared with cases. The mean number of full-term pregnancies was slightly higher in controls than in cases, and the mean age at first live birth was slightly older in controls than in cases, by an average of 1.4 years (Table 1).

Telomere length correlations between chromosome arms and age

We evaluated whether the telomere length was correlated between chromosomal arms in control subjects and found that lengths between homologous telomeres were significantly correlated: Spearman's correlation coefficients (r) ranged from 0.36 to 0.61 for 46 pairs of homologous telomeres (mean $r = 0.51$), all P-values < 0.0001 (highly significant after Bonferroni's correction for multiple comparisons 0.05/46 = 0.0011). In contrast, telomere lengths between non-homologous telomeres were not correlated, with r between -0.21 and 0.19 (only 21 out of 2116 correlations had r either < -0.15 or > 0.15, none of the P-values reached significance after the adjustment for multiple comparisons). We also examined correlations between arm-specific telomere lengths and the subject's age in control subjects. Significant correlations were observed for chromosome 15p-S ($r = -0.24$, $P = 0.0003$). Age was weakly correlated with within-cell telomere length variation (WCTLV) ($r = 0.20$, $P = 0.003$) and with total telomere length ($r = -0.15$, $P = 0.02$).

Arm-specific telomere length and breast cancer risk

Arm-specific relative telomere length (RTL) was defined as the percent of the arm-specific telomere fluorescent intensity units (FIU) divided by the total telomere FIU of 92 telomeres from the same cell. Initial case–control comparisons of mean RTLs identified four telomeres (1p-S, Xp-S, 9p-S and 15p-S) that showed case–control differences at $P \leq 0.01$, and none of

Table 1. Distribution of characteristics of study subjects and known breast cancer risk factors

	Cases, $n = 204$	Controls, $n = 236$	P-value
Demographic factors[a]			
Age (years)	53.0 ± 11.0	53.2 ± 10.0	0.85
Race (%)			
White	74.1	71.5	
Black	20.3	25.0	
Others	5.6	3.5	0.34
Education $\geq$ college (%)	40.9	43.4	0.61
Household income $\geq$ 100 K (%)	55.0	56.1	0.85
Reproductive risk factors[a]			
Age at menarche (years)	12.6 ± 1.5	12.5 ± 1.8	0.58
Post-menopausal (%)	55.3	57.9	0.59
Number of FTP[b]	1.65 ± 1.23	1.87 ± 1.12	0.09
Age at first FTP (years)	27.2 ± 6.4	28.6 ± 6.6	0.08
Used HRT[c] (%)	32.8	39.8	0.14
Other risk factors[a]			
Had FHC (%)	57.8	52.3	0.27
Body mass index	27.3 ± 6.5	27.3 ± 7.1	0.97
Ever smoked cigarettes (%)	37.6	46.2	0.07
Ever drank alcohol (%)	88.7	92.0	0.25
Exercised regularly at teens (%)	66.5	80.9	<0.001

Exercised regularly was defined as any weekly physical activity that would make the subject sweat or increase their heart rate and last $>$20 min. Family history of cancer (FHC) was defined as any cancer cases among first degree blood relatives.
[a]Unless otherwise specified, mean $\pm$ SD are presented.
[b]FTP, full-term pregnancies.
[c]HRT, hormonal replacement therapy.

the 46 telomeres showed case–control differences at the $P \leq 0.0005$ level [Bonferroni's correction for multiple comparisons $0.05/(46 \times 2) = 0.0005$] in pre-menopausal women. In post-menopausal women, one telomere (15p-S) showed case–control differences at $P \leq 0.01$, and none of the 46 telomeres showed case–control differences at the $P \leq 0.0005$ level (Table 2). It is important to note that none of the homologous long version of the telomeres showed noticeable case–control differences (Supplementary Material, Table S1). Because telomere lengths between homologous telomeres are significantly correlated, subsequent analyses were focused on the homologous short version of each of the 46 telomeres. Using the 50th percentile value in controls as a cut point, multivariate logistic regression analysis confirmed that short telomere lengths on Xp-S and 15p-S were associated with an increased breast cancer risk in pre-menopausal women: adjusted odds ratio (aOR) = 2.5 (95% CI = 1.3–4.8) and 2.6 (95% CI = 1.3–5.0), respectively (Table 3), which took into account the age, race, education, household income, physical activity in teens, smoking status, alcohol use and family history of cancer. When the study subjects were categorized into four groups (by quartiles) according to the telomere length, an inverse dose–response relationship was observed for Xp-S ($P_{trend} = 0.001$) and 15p-S ($P_{trend} = 0.004$), with the lowest versus highest quartile aOR (95% CI) of 5.5 (2.0–15.1) and 3.6 (1.4–9.8), respectively (Table 3). In post-menopausal women, multivariate logistic regression analysis revealed that the short telomere length on 15p-S was associated with a decreased breast cancer risk,

Table 2. Case–control comparison of mean RTL on homologous short version of chromosome arms

Chromosome arms	All subjects			Pre-menopausal women			Post-menopausal women		
	Cases, $n = 204$	Controls, $n = 236$	P-value[†]	Cases, $n = 89$	Controls $n = 96$	P-value[†]	Cases, $n = 110$	Controls, $n = 132$	P-value[†]
1p	0.75 (0.13)	0.78 (0.15)	0.04	0.74 (0.12)	0.81 (0.15)	0.003	0.75 (0.14)	0.76 (0.15)	0.65
Xp	0.78 (0.16)	0.80 (0.15)	0.29	0.76 (0.15)	0.83 (0.15)	0.001	0.78 (0.17)	0.78 (0.15)	0.31
9p	0.66 (0.14)	0.69 (0.13)	0.05	0.67 (0.14)	0.72 (0.13)	0.007	0.66 (0.14)	0.67 (0.13)	0.70
15p	0.70 (0.15)	0.70 (0.14)	0.64	0.68 (0.15)	0.74 (0.15)	0.013	0.71 (0.15)	0.66 (0.13)	0.01

RTL was defined as the percent of arm-specific telomere length divided by total telomere length of 92 telomeres. Mean (SD)s were presented in the table. P-values were considered significant if $P \leq 0.0005$ (Bonferroni's correction $0.05/(46 \times 2) = 0.0005$).
[†]P-values were based on Wilcoxon's rank-sum test.

aOR (95% CI) = 0.54 (0.3–0.9). A dose–response relationship was also observed for 15p-S ($P_{trend} = 0.004$, Table 3).

Telomere length variation between homologous telomeres and breast cancer risk

Because greater telomere length variation among chromosomal telomeres is one of the characteristics of cancer cells that use alternative lengthening of telomeres (ALT) to maintain the telomere length (17,18), we hypothesized that greater telomere length variation between homologous telomeres confers telomere instability, hence increased cancer risk. To test this hypothesis, we examined whether differences in length between homologous telomeres are associated with the breast cancer risk. The homologous telomere length difference (HTLD) was defined as a percent of (homologous long RTL − homologous short RTL) divided by (homologous long RTL + homologous short RTL). Initial case–control comparisons of the mean HTLD identified seven chromosome arms (5q, Xp, 8q, 9p, 12p, 15p and 15q) that showed case–control differences at the P-value ≤ 0.01 level, and two chromosome arms (9p and 15p) showed case–control differences at the P-value ≤ 0.0005 level [significant after Bonferroni's correction for multiple comparisons $0.05/(46 \times 2) = 0.0005$] in pre-menopausal women (Table 4). None of the 46 chromosome arms showed significant case–control differences in post-menopausal women (Supplementary Material, Table S2). Using the 50th percentile value in controls as a cut point, multivariate logistic regression analysis confirmed that greater HTLD on chromosome arms 9p, 15p and 15q was associated with an increased breast cancer risk in pre-menopausal women, aOR (95% CI) = 4.6 (2.3–9.2), 3.1 (1.6–6.0) and 2.8 (1.4 to 5.4), respectively (Table 5). When the study subjects were categorized into four groups (by quartiles) according to the HTLD, a dose–response relationship was observed for chromosomes Xp ($P_{trend} = 0.005$), 9p ($P_{trend} < 0.001$), 15p ($P_{trend} < 0.001$) and 15q ($P_{trend} = 0.005$), respectively (Table 5). None of the chromosome arms showed significant association with the breast cancer risk in post-menopausal women (Table 5).

Telomere length variations and breast cancer risk

To test the hypothesis that increased telomere length variations will increase genomic instability, and hence increased

risk of breast cancer, we examined if WCTLV, defined as coefficient of variation (CV) of the RTLs among 46 non-homologous chromosome arms (combining the telomere lengths between the two homologous arms) within a cell, is associated with the breast cancer risk. We found that mean WCTLV was higher in cases (mean = 46.2%) than in controls (mean = 44.6%, $P = 0.006$) in pre-menopausal women. Using the 50th percentile value in controls as a cut point, multivariate logistic regression analysis confirmed that greater WCTLV was associated with an increased breast cancer risk in pre-menopausal women, aOR (95% CI) = 1.9 (1.0–3.7). When the study subjects were categorized into four groups (by quartiles) according to the WCTLV, a dose–response relationship was also observed ($P_{trend} = 0.012$) with aOR (95% CI) of 1.6 (0.6–4.0), 1.9 (0.8–4.7) and 3.7 (1.3–10.1) for second, third and fourth quartiles, respectively. We observed no association between WCTLV and breast cancer risk in post-menopausal women (Supplementary Material, Table S3).

DISCUSSION

In this report, we showed that, after adjustment for known breast cancer risk factors, shorter telomere lengths on chromosomes Xp and 15p were associated with an increased risk of breast cancer in pre-menopausal women. These data support our hypothesis that women who have telomere length deficiency on specific chromosome arms are at an increased risk of breast cancer. Our results are consistent with the concept by previous reports showing that telomere dysfunction plays a role in breast cancer development (19–21).

A key feature necessary for telomere integrity is the maintenance of telomeric DNA at a critical length that allows assembly of the protective end structures. Current knowledge indicates that the telomere length and structure are tightly regulated by several distinct but cooperative mechanisms: (i) replenishment of telomere DNA by telomerase (a ribonucleo-protein reverse transcriptase) (22); (ii) HR-mediated telomere maintenance or ALT (23); (iii) length regulation through epigenetic modification of telomeric and subtelomeric DNA (24); and (iv) negative regulation of the telomere length by a trimming mechanism (25). Telomeres are thought to play a key role in tumor suppression by limiting the number of times a cell can divide, even in the presence of oncogenic mutations. Deficiency in telomere length maintenance is particularly

Table 3. Logistic regression examining the association between homologous short RTL and breast cancer risk

Chromosome arms	All subjects, $n = 440$ OR (95% CI)	*P*-value	Pre-menopausal women, $n = 185$ OR (95% CI)	*P*-value	Post-menopausal women, $n = 242$ OR (95% CI)	*P*-value
1p						
By median	1.35 (0.89–2.03)	0.16	1.73 (0.90–3.31)	0.10	1.08 (0.62–1.87)	0.79
By quartiles						
Q3	1.14 (0.63–2.05)		1.97 (0.80–4.84)		0.75 (0.33–1.71)	
Q2	1.15 (0.64–2.08)		1.96 (0.76–4.95)		0.72 (0.32–1.60)	
Q1	1.76 (0.99–3.13)	0.06*	3.07 (1.20–7.86)	0.026*	1.15 (0.54–2.45)	0.65*
Xp						
By median	1.12 (0.75–1.67)	0.59	2.50 (1.31–4.78)	0.006	0.59 (0.34–1.02)	0.06
By quartiles						
Q3	1.61 (0.90–2.86)		3.38 (1.31–8.69)		0.90 (0.40–2.02)	
Q2	1.17 (0.64–2.12)		5.21 (1.84–14.8)		0.37 (0.16–0.85)	
Q1	1.64 (0.92–2.91)	0.21*	5.45 (1.97–15.1)	0.001*	0.69 (0.32–1.49)	0.15*
9p						
By median	1.13 (0.75–1.69)	0.56	1.42 (0.75–2.69)	0.28	1.02 (0.59–1.78)	0.94
By quartiles						
Q3	2.18 (1.21–3.91)		3.14 (1.28–7.72)		1.55 (0.68–3.54)	
Q2	1.55 (0.84–2.84)		2.23 (0.89–5.61)		1.27 (0.54–2.98)	
Q1	1.88 (1.04–3.41)	0.14*	2.54 (1.00–6.43)	0.09*	1.37 (0.61–3.08)	0.65*
15p						
By median	1.05 (0.70–1.57)	0.83	2.56 (1.32–4.97)	0.005	0.54 (0.31–0.94)	0.028
By quartiles						
Q3	0.75 (0.42–1.33)		1.64 (0.67–4.03)		0.38 (0.17–0.87)	
Q2	1.02 (0.58–1.77)		2.99 (1.23–7.26)		0.41 (0.18–0.91)	
Q1	0.82 (0.46–1.44)	0.72*	3.63 (1.35–9.75)	0.004*	0.30 (0.14–0.64)	0.004*

ORs were adjusted for age, race, education, household income, physical activity in teens, smoking status, alcohol use and family history of cancer. Q4 is the referent group for computing the ORs.
P-for-trend.

relevant to carcinogenesis because hyper-proliferative cells could lead to progressive telomere shortening, ultimately generating uncapped telomeres that fuse with each other and leading to genomic instability that promotes malignant transformation. However, it is unclear whether it is the shortest telomeres or the mean telomere length that triggers the telomere dysfunction-associated responses. Several lines of evidence favor the concept that a subset of shortest telomeres triggers the telomere dysfunction-associated responses. First, it has been shown that individual dysfunctional telomeres are detected by the cells as DNA damage, triggering a cellular response (26). Crossing telomerase knockout mice having short telomeres with those having long telomeres revealed that the loss of telomere function occurs preferentially on the shortest telomere and that the shortest telomeres, rather than the average telomere length, elicit a cellular response (27). Second, several studies demonstrated that chromosome arms carrying the shortest telomeres were more often found in telomere fusions, leading to chromosomal instability (28–30). Third, in humans, chromosome-specific telomere lengths are highly variable between chromosomal arms (31–33). The potential implication of this chromosome arm-specific telomere length polymorphism is that chromosome arms bearing the shortest telomeres may predispose to the chromosome alterations and therefore have an impact on the evolution of tumors. Given such previous evidence, it is surprising that so little research has investigated the length of individual telomeres and its relationship to human disease. So far, there are only two recent small studies examined association between telomere lengths on a few chromosome arms and the risk of breast (34) and esophageal (35)

cancers. The short telomere length on chromosome 9p was found to be significantly associated with the breast cancer risk (34) and short telomeres on chromosomes 17p and 12q were found to be significantly associated with an increased risk of esophageal cancer (35). To the best of our knowledge, the present study is the first that examined the association between all 92 individual telomeres in the human genome and risk of breast cancer. Our data suggest that short telomere lengths on chromosomes Xp and 15p are associated with the breast cancer risk in pre-menopausal women. Our data also revealed that telomere lengths between non-homologous telomeres were not correlated and are likely independent telomere-associated parameters that may carry information of clinical importance for cancer patients.

The comprehensive telomere analysis approaches used in the present study allowed us to examine the associations between telomere length variations and breast cancer risk. Since homogenous protection for all chromosome ends is required to prevent telomere dysfunction, we hypothesized that a high degree of telomere length variation represents a deficiency in telomere maintenance that is related to cancer susceptibility. Our data indicated that greater telomere length differences between homologous telomeres on chromosomes 9p, 15p and 15q were associated with the breast cancer risk in pre-menopausal women. To the best of our knowledge, the present report is the first to introduce HTLD as a novel phenotype of telomere deficiency and to have identified greater HTLD on chromosomes 9p, 15p and 15q as potential new risk factors for breast cancer in pre-menopausal women.

The present study also revealed that WCTLV among 46 non-homologous telomeres is associated with the breast

Table 4. Case–control comparison of mean HTLDs

Chromosome arms	All subjects Cases, $n = 204$	Controls, $n = 236$	P-value	Pre-menopausal women Cases, $n = 89$	Controls, $n = 96$	P-value	Post-menopausal women Cases, $n = 110$	Controls, $n = 132$	P-value
5q	38.5 (7.6)	36.2 (6.9)	0.006	39.4 (7.6)	35.6 (7.1)	0.003	37.9 (7.5)	36.6 (6.7)	0.21
Xp	38.3 (7.9)	37.2 (7.3)	0.09	39.4 (8.3)	36.4 (6.4)	0.010	37.6 (7.7)	37.9 (7.9)	0.98
8q	40.7 (8.0)	38.5 (7.8)	0.010	40.9 (7.8)	38.0 (7.0)	0.013	40.8 (8.2)	38.9 (8.4)	0.11
9p	38.9 (7.6)	37.1 (7.6)	0.015	39.1 (7.4)	35.9 (7.2)	**0.0005**	38.9 (7.8)	38.1 (7.7)	0.65
12p	38.8 (7.7)	36.9 (7.5)	0.016	39.8 (8.2)	36.5 (7.5)	0.009	38.2 (7.3)	37.3 (7.5)	0.46
15p	38.9 (7.4)	38.2 (7.9)	0.30	40.1 (7.8)	35.9 (7.1)	$\mathbf{9 \times 10^{-5}}$	38.1 (7.1)	40.0 (8.1)	0.04
15q	38.1 (7.4)	36.5 (7.7)	0.021	38.2 (7.6)	34.9 (7.5)	0.003	38.2 (7.3)	38.0 (7.5)	0.74

HTLD was defined as the percent of (homologous long RTL − homologous short RTL) divided by (homologous long RTL + homologous short RTL). Mean (SD)s were presented in the table. P-values were based on Wilcoxon's rank-sum test. Bold P-values are significant after adjustment for multiple comparisons (Bonferroni's correction $0.05/(46 \times 2) = 0.0005$).

cancer risk in pre-menopausal women. These data provided further new evidence that greater telomere length heterogeneity may contribute to an increased breast cancer risk in pre-menopausal women. A substantial number of human malignant tumors utilize ALT, a telomerase-independent telomere length maintenance mechanism that involves HR, to maintain telomere length. These include bone and soft tissue sarcomas, glioblastomas and carcinomas of the lung, kidney, breast and ovary (17,18). ALT-mediated telomere length maintenance is characterized by highly heterogeneous telomeric DNA length (36,37). Within an ALT+ cell, there are telomeres that ranged from <2 kb to >50 kb in length, and often several chromosome ends lacking any telomere signal (37). Given the important role of HR in normal telomere biology, it is possible that dysregulation of HR-assisted telomere maintenance may result in increased telomere length variations between individual telomeres in somatic cells, resulting in increased risk of cancer. Additionally, recent data indicated that the telomere length is negatively regulated by a mechanism that trims telomeric repeats from the chromosome ends, preventing excessive long telomeres (25), indicating that telomere lengths are carefully regulated by multiple mechanisms to maintain the telomeres at an 'optimal' length (not too long, not too short), essential for normal telomere homeostasis. Therefore, increased telomere length variation is likely representing deficiencies in certain aspect of telomere regulation machinery, leading to telomere instability and increased cancer risk. Our discoveries that greater telomere length variations between homologous telomeres and across all chromosome ends in a cell are associated with an increased risk of breast cancer in pre-menopausal women provided first evidence to support this hypothesis.

Our data indicated that the associations between telomere deficiencies and breast cancer risk in pre-menopausal women only involve a handful of chromosome arms (Xp, 9p, 15p and 15q). The reason why these chromosomal arms were involved is unknown, possibly related to telomere-mediated dysregulation of genes that reside on those chromosome arms and that are also involved in breast carcinogenesis. For example, telomere lengths are shown to be the critical players in regulating epigenetic modification of regional chromatin and these telomere-related epigenetic changes could result in epigenetic dysregulation of oncogenes and/or tumor

suppressor genes (38–40). Deficiency in 9p telomeres could potentially affect the stability of chromosome 9p, where the *CDKN2A* locus (also known as the *INK4a/ARF* locus) locates at 9p21. The *CDKN2A* locus encodes two proteins, $p16^{INK4a}$ and $p14^{ARF}$, that regulate two critical cell cycle regulatory pathways: the p53 pathway and the retinoblastoma pathway (41,42). Inactivation of the *CDKN2A* locus removes an important barrier to tumor progression and 9p21 is a frequent target of inactivation by deletion or aberrant DNA methylation in a wide variety of human cancers (43), including breast cancer (44–46). Despite its importance in tumor suppression and considerable research, the cause of *CDKN2A* inactivation by deletion or aberrant promoter methylation is currently unknown. To the best of our knowledge, no study has investigated whether chromosome arm-specific telomere dysfunction induces inactivation of specific tumor suppressor genes or amplification of oncogenes in human or model organisms. However, these are important biological questions that could reveal a critical step in carcinogenesis, thus warranting further investigation.

Results from the present study indicated that the association between chromosome arm-specific telomere deficiencies and breast cancer risk is restricted to pre-menopausal women. In post-menopausal women, there is only a suggestive association between the short telomere length on chromosome 15p and a decreased breast cancer risk. However, it should be noted that none of the associations in post-menopausal women was statistically significant after considering correction for multiple comparisons. Thus, future larger studies are needed to define the role of telomeres in breast cancer among post-menopausal women.

Given that this is a case–control study, a theoretical concern is that the telomere length in lymphocytes is affected by case status (reverse causality). Data by previous studies and by us indicated that the mean overall telomere length of blood leukocytes in breast cancer patients was not significantly shorter than in healthy women controls (11,12,14,15), suggesting there is no significant shortening of blood leukocyte telomere length associated with having breast cancer. Although previous studies (47) suggested that chemotherapy and/or radiotherapy can induce telomere shortening in leukocytes, all the blood samples in our study were drawn before any chemotherapy and radiotherapy treatments. Thus, reverse causality is not a plausible explanation for our

Table 5. Logistic regression examining the association between HTLD and breast cancer risk

Chromosome arms	All subjects, $n = 440$ OR (95% CI)	P-value	Pre-menopausal women, $n = 185$ OR (95% CI)	P-value	Post-menopausal women, $n = 242$ OR (95% CI)	P-value
5q						
By median	1.56 (1.03–2.36)	0.036	1.92 (1.00–3.71)	0.05	1.47 (0.84–2.58)	0.18
By quartiles						
Q2	1.10 (0.63–1.89)		1.21 (0.51–2.83)		0.97 (0.46–2.04)	
Q3	1.49 (0.84–2.64)		1.41 (0.60–3.34)		1.75 (0.79–3.91)	
Q4	1.87 (1.05–3.32)	0.022*	3.43 (1.32–8.88)	0.017*	1.31 (0.61–2.85)	0.28*
Xp						
By median	1.31 (0.87–1.97)	0.19	1.82 (0.95–3.49)	0.07	1.08 (0.63–1.88)	0.77
By quartiles						
Q2	1.24 (0.71–2.18)		2.94 (1.15–7.55)		0.64 (0.30–1.38)	
Q3	1.24 (0.71–2.17)		2.16 (0.87–5.37)		0.84 (0.39–1.81)	
Q4	1.81 (1.02–3.21)	0.06*	3.98 (1.63–9.70)	0.005*	0.89 (0.40–2.00)	0.94*
8q						
By median	1.37 (0.91–2.06)	0.13	1.63 (0.87–3.05)	0.13	1.26 (0.73–2.19)	0.41
By quartiles						
Q2	2.53 (1.41–4.54)		4.34 (1.72–10.92)		1.70 (0.77–3.80)	
Q3	1.65 (0.95–2.88)		3.03 (1.19–7.75)		1.17 (0.57–2.40)	
Q4	2.37 (1.35–4.16)	0.008*	3.57 (1.44–8.84)	0.015*	2.00 (0.94–4.24)	0.13*
9p						
By median	1.92 (1.26–2.92)	0.002	4.59 (2.29–9.20)	<0.001	1.07 (0.61–1.88)	0.83
By quartiles						
Q2	0.94 (0.55–1.62)		1.22 (0.46–3.21)		1.03 (0.50–2.12)	
Q3	1.82 (1.01–3.28)		7.18 (2.48–20.79)		0.83 (0.38–1.80)	
Q4	1.98 (1.09–3.58)	0.005*	4.29 (1.53–11.99)	<0.001*	1.45 (0.65–3.16)	0.54*
12p						
By median	1.25 (0.83–1.87)	0.29	2.01 (1.06–3.84)	0.033	0.86 (0.50–1.49)	0.59
By quartiles						
Q2	1.21 (0.70–2.11)		1.31 (0.54–3.18)		1.10 (0.51–2.36)	
Q3	1.28 (0.73–2.24)		2.29 (0.91–5.74)		0.82 (0.38–1.75)	
Q4	1.46 (0.83–2.58)	0.19*	2.25 (0.94–5.40)	0.037*	1.00 (0.46–2.20)	0.81*
15p						
By median	1.18 (0.78–1.78)	0.43	3.06 (1.58–5.95)	0.001	0.58 (0.33–1.01)	0.05
By quartiles						
Q2	0.78 (0.45–1.37)		1.32 (0.50–3.45)		0.64 (0.30–1.36)	
Q3	0.91 (0.51–1.63)		2.56 (0.99–6.64)		0.48 (0.22–1.04)	
Q4	1.12 (0.63–1.99)	0.62*	4.44 (1.70–11.6)	<0.001*	0.42 (0.19–0.95)	0.023*
15q						
By median	1.58 (1.05–2.38)	0.030	2.79 (1.44–5.40)	0.002	1.16 (0.66–2.03)	0.62
By quartiles						
Q2	1.07 (0.62–1.84)		1.09 (0.42–2.83)		0.90 (0.44–1.80)	
Q3	1.62 (0.91–2.88)		2.91 (1.17–7.27)		1.12 (0.51–2.48)	
Q4	1.65 (0.92–2.95)	0.042*	2.89 (1.17–7.16)	0.005*	1.05 (0.46–2.38)	0.78*

ORs were adjusted for age, race, education, household income, physical activity in teens, smoking status, alcohol use and family history of cancer. Q1 is the referent group for computing the ORs. HTLD was defined as the percent of (homologous long RTL − homologous short RTL) divided by (homologous long RTL + homologous short RTL).
*P-for-trend.

observed associations. Our study is limited by its moderate sample size and does not have sufficient statistical power to detect any small to moderate associations (i.e. OR < 2.0). After Bonferroni's correction for multiple comparisons, observed case–control differences were only borderline significant for the HTLD on chromosomes 9p and 15p (Table 4). Thus, future larger studies in independent populations are needed to confirm our findings and to generate more precise estimation on risk association.

In summary, the present study revealed that the short telomere length on chromosomes Xp and 15p, greater length differences between homologous telomeres on chromosomes 9p, 15p and 15q and greater WCTLV were associated with an increased risk of breast cancer in pre-menopausal women. Our data also suggest that telomere length variations between homologous

telomeres or within a somatic cell are likely distinct phenotypes of telomere deficiencies. These new discoveries have potentially important clinical implications. If confirmed in future studies, telomere-related parameters are likely to be a useful panel of blood-based biomarkers for breast cancer risk assessment, given their strong associations with the breast cancer risk. These data also provided new clues on the possible role of telomeres in breast carcinogenesis.

MATERIALS AND METHODS

Study population

The study was approved by the MedStar Research Institute-Georgetown University Oncology Institutional Review

Board. The details of study population were described previously (34). The inclusion criteria for cases included a diagnosis of female breast cancer within the prior 6 months, have not been treated yet with chemotherapy and radiotherapy, and ability to provide informed consent in English. Exclusion criteria were women with a prior history of cancer, had chemotherapy and/or radiation treatment or had active infection or immunological disorder being treated with antibiotics or immunosuppressive medication within the prior 1 month. The participation rate among eligible patients was 70%.

Controls were randomly selected from healthy women who visited the mammography screening clinic at Georgetown University Medical Center, frequency matched to cases by age (2-year interval), race and state of residency (D.C., Maryland or Virginia). Other inclusion and exclusion criteria for controls were the same as for cases. Additionally, women who had a breast biopsy, pregnant or breast feeding were not eligible. The participation rate among the eligible women was 60% for controls.

After providing informed consent, all subjects received a structured, in-person interview assessing prior medical history, tobacco smoke exposures, alcohol use, current medications, family medical history, reproductive history and socioeconomic characteristics. Venous blood was obtained by trained interviewers using heparinized tubes.

Telomere length measurement and quality control

Chromosome arm-specific telomere lengths were measured by telomere quantitative fluorescent *in situ* hybridization (TQ-FISH). Chromosome preparations were obtained using short-term lymphocyte cultures that were established from fresh blood, as previously described (48). The chromosome preparations were dropped onto clean microscopic slides and hybridized with 15 μl of hybridization mixture consisting of 0.3 μg/ml Cy3-labeled telomere-specific peptide nucleic acid (PNA) probe, 1 μl of cocktails of FITC-labeled centromeric PNA probes specific for chromosomes 2, 4, 8, 9, 13, 15, 18, 20 and 21, 20 μg/ml of Cy3-labeled centromeric PNA probes specific for chromosome X (Biomarkers, Rockville, MD, USA), 50% formamide, 10 mM Tris–HCl, pH 7.5 and 5% blocking reagent. Slides were then placed in a Hybex microarray hybridization oven where the DNA was denatured by incubating at 75°C for 5 min, followed by hybridizing at 30°C for 3 h. After hybridization, the slides were sequentially washed 10 min each at 42°C: once in 1 × SSC, once in 0.5 × SSC and once in 0.1 × SSC. The slides were then mounted in anti-fade mounting medium containing 300 ng/ml 4′-6-diamidino-2-phenylindole (DAPI).

After TQ-FISH, cells were analyzed using an epifluorescence microscope equipped with a charge-coupled device camera. Images were captured with exposure times of 0.15, 0.25 and 0.05 second for Cy3, FITC and DAPI signals, respectively. Digitized images were analyzed using a specialized Isis FISH imaging software with a telomere module (MetaSystems Inc., Boston, MA, USA). This software permits measurement of 92 telomere signals simultaneously after karyotyping (Supplementary Material, Fig. S1). Chromosome identification was achieved by: (i) DAPI banding (equivalent to G-banding, Supplementary Material, Fig. S1a); and

(ii) chromosome-specific centromere probes (Supplementary Material, Fig. S1b). Telomere FIU were recorded as an indirect measurement of telomere length. We noticed that between the homologous telomeres, one telomere was often shorter than the other and there are noticeable differences in lengths between homologous telomeres. Thus, each pair of homologous telomeres was recorded separately as homologous short (S) and homologous long (L). To normalize the FISH hybridization variations between samples, RTL, defined as the percent of the arm-specific telomere FIU divided by the total telomere FIU of 92 telomeres from the same cell, was used for the subsequent statistical analysis. For each study subject, 15 metaphase cells were analyzed to estimate the mean RTL for each of the 92 telomeres.

Definitions of telomere-related parameters: (i) arm-specific RTL, defined as the percent of the arm-specific telomere FIU divided by the total telomere FIU of 92 telomeres from the same cell; (ii) the HTLD was defined as the percent of (homologous long RTL − homologous short RTL) divided by (homologous long RTL + homologous short RTL). The HTLD measures the telomere length variation between homologous telomeres; (iii) WCTLV, defined as the CV of the RTLs among 46 non-homologous chromosome arms (combining the telomere lengths between the two homologous arms) within a cell. WCTLV measures the overall variation of telomere length distribution among 46 telomeres in a cell.

Several quality control steps were implemented in the telomere assay. Laboratory personnel who were responsible for the blood culture and telomere assay were blinded to the case–control status of the subjects. All new lots of reagents were tested to ensure optimal hybridization. A control slide containing cells with known total telomere length was included in each batch of TQ-FISH to monitor the quality of the hybridization efficiency. Case and control samples were analyzed together in each batch and a total of 13 batches were run for the whole case–control set. The QC data from 13 repeated TQ-FISH experiments of the control cells were as following: the mean CV of arm-specific RTLs, HTLD and WCTLV were 12.6, 12.4 and 4.9%, respectively.

Statistical analysis

Wilcoxon's rank-sum test was used to compare the means of the continuous variables. Chi-square tests were used to compare the distribution of categorical variables between cases and controls. Spearman's correlation was used to examine the correlations between telomere lengths, and between telomere lengths and age.

The associations between telomere-related parameters and the risk of breast cancer were examined using unconditional logistic regression. RTLs, HTLD and WCTLV were dichotomized as short/long using the 50th percentile values in the controls as a cut point. Telomere-related parameters were also categorized according to the quartiles in control subjects. ORs were adjusted for age, race, smoking status, alcohol use, education, family history of cancer in the first degree biological relatives, menopausal status and physical activity in the teens. *P*-values were two-sided and considered statistically significant after adjustment for multiple comparisons (Bonferoni's correction at $\alpha \leq 0.05$ level). All analyses were

performed using SAS software, version 9 (SAS Institute Inc., Cary, NC, USA).

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