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Chromosome 9 arm-specific telomere length and breast cancer risk

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Background: **Telomere dysfunction is involved in the development of breast cancer and very short telomeres are frequent genetic alterations in breast tumors. However, the influence of telomere lengths of specific chromosomal arms on the breast cancer risk is unknown.** ***Methods:*** **We conducted a case–control study of breast cancer to examine the associations of the telomere length on chromosome 9 short arms (9p) and long arms (9q) with risk of breast cancer. Chromosome 9 arm-specific telomere lengths were measured by quantitative fluorescent *in situ* hybridization using cultured blood lymphocytes.** ***Results:*** **Telomere length on chromosome 9p was significantly shorter in breast cancer patients than in control subjects ($P < 0.001$). Using the 50th percentile value in controls as a cut point, women who have short 9p telomeres had an increased risk of breast cancer [adjusted odds ratio (OR) = 2.6; 95% confidence interval (CI) = 1.5–4.3]. When the 9p telomere length was divided into quartiles, a significant inverse dose–response relationship between 9p telomere length and breast cancer risk was observed ($P_{trend} < 0.001$), with a quartile ORs of 3.0 (95% CI = 1.2–7.5), 3.9 (95% CI = 1.6–9.5) and 6.6 (95% CI = 2.8–15.9) for third, second and first quartile, respectively, when compared with women in the fourth quartile.** ***Conclusions:*** **Short telomere length on chromosome 9p is strongly associated with the risk of breast cancer. If confirmed by future studies, chromosome 9p telomere length has the potential to be incorporated into the current prediction models to significantly enhance breast cancer risk prediction.**

Introduction

Breast cancer is the most common malignancy in women (1). In the USA, breast cancer incidence rates have been rising slowly for the past two decades, and breast cancer is the second leading cause of cancer-related death in women (2,3). However, there is currently no accurate method to predict who is most probably to develop the disease for individuals in the general population. Of the nearly 241 000 women diagnosed each year, ~80 to 90% are sporadic cases who had no family history of breast cancer and no other identifiable strong risk factors other than age and reproductive or hormonal risk factors (4). In order to prevent breast cancer, there is a need to develop tools to identify women at an elevated risk, allowing women and their physicians to take a more proactive approach to reduce breast cancer burden.

Breast cancer, like most human malignancies, is characterized by telomere dysfunction and chromosomal instability (5–7). It is well documented that chromosomal instability preferentially involves specific chromosome arms for each type of human cancer (5). In breast cancer, frequent chromosomal abnormalities in early stage breast tumors involves a few chromosomal arms, including gains of 1q, 8q, 17q and 20q, and losses of 8p, 9p, 16q and 17p (5,8,9). Chromosome arm-specific telomere deficiency could be one of the underlying mechanisms for such arm-specific instability because critically short/dysfunctional telomeres can lead to chromosome end fusion that induces chromosome-specific instability via repeated series of breakage-fusion-bridge cycles (10–13). Telomeres are specialized DNA–protein structures that cap the ends of linear chromosomes. They are crucial for protecting linear chromosomes and are essential for maintaining the integrity and stability of genomes (14). Telomere-induced chromosomal instability could drive the tumorigenic process by increasing mutation rates for oncogenes and tumor suppressor genes (15).

Accumulating evidence indicates that dysregulation of the p53 and retinoblastoma (Rb) pathways are important mechanisms of chromosome instability (16), and deregulation of the Rb pathway is a major contributor to chromosomal instability in breast tumors (8,17). One of the chromosomal abnormalities that affects the regulation of both p53 and Rb pathways is the chromosome 9p21 deletion. Indeed, deletions of chromosome 9 or 9p21 are the most frequent early chromosomal abnormalities in human cancers, including head and neck (18), bladder (19), non-small cell lung (20) and skin cancers (5,21). Frequent chromosome 9p deletions were also reported for high-grade ductal carcinoma *in situ* (22,23) and invasive breast cancer (9,24). On 9p, deletions and recombination are centered around an important tumor suppressor locus, the *CDKN2A* (25,26). The *CDKN2A* gene encodes two proteins ($p16^{INK4}$ and $p14^{ARF}$) that regulate two critical cell cycle regulatory pathways: the p53 pathway and the Rb pathway (27). Thus, inactivation of the CDKN2A locus via chromosome 9p21 deletion may be an initiating event in the development of breast cancer. We hypothesized that chromosomes possessing short telomeres are unstable and so individuals who have short telomeres on chromosome 9 will have an increased likelihood of chromosome 9 deletion and consequently a greater risk to develop cancer. To test this hypothesis, we conducted a case–control study of breast cancer, examining the association between chromosome 9 arm-specific telomere lengths and breast cancer risk. We demonstrated that short telomere length on chromosome 9p is strongly associated with breast cancer risk and could potentially become a novel tool for breast cancer risk assessment for individuals and populations.

Abbreviations: CI, confidence interval; FITC, fluorescein isothiocyanate; OR, odds ratio; Rb, retinoblastoma; RTL, relative telomere length.

Materials and methods

Study population

Breast cancer cases ($n = 153$) were recruited at the Georgetown University Hospital clinics (Lombardi Comprehensive Cancer Center's Division of Medical Oncology, Department of Surgery and the Betty Lou Ourisman Breast Cancer Clinic). The inclusion criteria for cases included a diagnosis of breast cancer within the prior 6 months, women who have not been treated yet with chemotherapy and radiotherapy, and are able to provide informed consent in English. Exclusion criteria were women with a prior history of cancer, had chemotherapy and radiation treatment or had active infection or immunological disorder that needed to be treated with antibiotics or immunosuppressive medication within the prior 1 month. From 2006 through 2008, a total of 228 newly diagnosed breast cancer patients were identified to be eligible and 153 (67%) participated in our study. The common reasons for non-participation were too busy or not interested (24%), overwhelmed by cancer diagnosis (5%) and not responsive to phone call or e-mail contact (4%).

Controls ($n = 159$) were randomly selected from healthy women who visited the mammography screening clinic at Georgetown University Hospital, frequency matched to cases by age (two year interval), race and state of residency (DC, Maryland or Virginia). Other inclusion and exclusion criteria for controls were the same as for cases. Additionally, women who had breast biopsy or were pregnant or breast-feeding were not eligible. The overall participation rate among the eligible women was 57% for controls. The major reason for non-participation was being either too busy (19%) or not interested (23%).

After providing informed consent, 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. The study was approved by the MedStar Research Institute-Georgetown University Oncology Institutional Review Board.

Blood cultures and preparation of chromosome spreads

Lymphocyte cultures were prepared from fresh blood within 48 h after the samples were obtained, as described previously (28). An 1 ml of fresh whole blood was added to 9 ml of RPMI-1640 medium supplemented with 15% fetal bovine serum (Biofluids, Rockville, MD), 1.5% of phytohemagglutinin (Invitrogen Corporation, Carlsbad, CA), 2 mM L-glutamine and 100 U/ml each of penicillin and streptomycin. Cells were cultured for 4 days at 37°C and colcemid (0.2 μg/ml) was added to the culture 1 h before the harvest. The cells were treated in hypotonic solution (0.06 M KCl) at room temperature for 25 min, fixed in crayon fixative (methanol:acetic acid = 3:1) and kept in crayon fixative at −20°C.

Telomere quantitative fluorescent in situ hybridization

The chromosome preparations were dropped onto clean microscopic slides and kept at room temperature for 7 days. Slides were then fixed in crayon fixative for 1 h, dehydrated through an ethanol series (70, 80, 90 and 100%) and air-dried. Fifteen microliters of hybridization mixture consisting of 0.3 μg/ml Cy3-labeled telomere-specific peptide nucleic acid probe (Panagene, Daejeon, Korea), 30 ng/ml of fluorescein isothiocyanate (FITC)-labeled chromosome 9-specific probe (Biomarkers LLC, Rockville, MD), 50% formamide, 10 mM Tris–HCl, pH 7.5, 5% blocking reagent and ×1 Denhart's solution was applied to each slide. Slides were then placed in a Hybex microarray hybridization oven (SciGene, Sunnyvale, CA) 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; once in 1× SSC, once in 0.5× SSC and once in 0.1× SSC, each wash was 10 min at 42°C. The slides were then mounted in anti-fade mounting medium containing 300 ng/ml 4′-6-diamidino-2-phenylindole.

The slides were analyzed using an epifluorescence microscope equipped with a charge-coupled device camera (Leica Microsystems, Bannockburn, IL). Images were captured with exposure times of 0.15, 0.25 and 0.05 s for Cy3, FITC and 4′-6-diamidino-2-phenylindole signals, respectively. Digitized images (supplementary Figure 1 is available at *Carcinogenesis* Online) were quantified using a semi-automated script, TeloMeter (provided by Dr Alan Meeker), which was written with image analysis software (ImageJ). This software permits measurement of telomere signals in defined regions, i.e. single chromosome arm or a cell (6). Chromosome 9 was identified by a FITC-labeled chromosome 9-specific probe (supplementary Figure 1 is available at *Carcinogenesis* Online). Telomere length was expressed as fluorescent intensity units. We noticed that between the pair of chromosome 9 short arms (9p) or long arms (9q), one telomere is always shorter than the other and there are significant differences in lengths between allelic telomeres. This observation is consistent with previous reports, which indicated that arm-specific telomere lengths were highly variable between chromosome arms and between allelic arms (29,30). Thus, four telomeres from chromosome 9 were recorded separately and treated as separate parameters. For each patient, 15 metaphase spreads were analyzed to estimate the mean telomere length for the: (i) short version of chromosome 9p (9p-short); (ii) long version of chromosome 9p (9p-long); (iii) short version of chromosome 9q (9q-short); (iv) long version of chromosome 9q (9q-long)and (v) total telomere length of the cell. The relative telomere length (RTL) of chromosome 9 arms were defined as the ratio of the arm-specific telomere fluorescent intensity units to the total telomere fluorescent intensity units of the cell, thus effectively normalizing the hybridization variations among individual samples. The rationale to use total telomere signals as reference signal for the normalization are (i) the same probe was used to measure total telomere length as arm-specific telomere length, thus providing a most efficient normalization and (ii) total telomere length is not significantly associated with breast cancer risk in this study (31), which is also consistent with previous reports (32–34).

Several quality control steps were used in this 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 telomere quantitative fluorescent *in situ* hybridization to monitor the quality of the hybridization efficiency. In this study, the coefficient of variation of total telomere length from 20 repeats of the control slide was 12.4%.

Statistical analysis

The final sample size for case–control analysis is 140 cases and 159 controls. Thirteen cases were excluded due to no histological confirmation of breast cancer diagnosis ($n = 8$) and blood culture failure ($n = 5$). Student's t-test was used to compare the means of chromosome 9 telomere lengths between cases and controls because the normality test indicated that these variables were symmetrically distributed. Chi-square tests were used to compare the distribution of categorical variables between cases and controls. Pearson correlation was used to examine the correlations between chromosome 9 telomere lengths and age.

We examined the associations between chromosome 9 telomere lengths and the risk of breast cancer, using unconditional logistic regression. Telomere lengths were dichotomized as short/long using the 50th or 75th percentile values in the controls as a cut point. Telomere lengths were also categorized according to the quartiles in controls. Odds ratios (ORs) were adjusted for age, race, smoking status, alcohol use, education, history of cancer in first- or second-degree relatives, menopausal status and physical activity in the teens. P-values were two sided and considered statistically significant if $P < 0.05$. All analyses were performed using SAS software, version 9 (SAS Institute, Cary, NC).

Results

Characteristics of study population

Table I lists the characteristics of the study subjects. The mean age is 52.7 for cases and 53.3 for controls. There are no significant case–control differences in the distributions of race, menopausal status, tobacco smoking, alcohol use, education levels, family history of cancer and hormone replacement therapy use. The mean body mass index was similar between cases and controls. The mean total telomere length was not significantly different between cases and controls. Controls were significantly more probably to be physically active in both the teens and in the past year compared with cases. There were no significant differences in the distribution of the levels of household income between cases and controls among those who reported household income. However, 31% of the cases and 16% of the controls did not report household income (Table I).

Chromosome 9 telomere lengths by case–control status

Table II presents the case–control comparisons of the means of the four chromosome 9 telomeres (9p-short, 9p-long, 9q-short and 9q-long). The mean RTL of the 9p-short was significantly shorter in cases (mean = 0.515%) than in controls (mean = 0.593%, $P < 0.001$). The mean RTL of the 9p-long was also significantly shorter in cases (mean = 1.184%) than in controls (mean = 1.234%, $P = 0.017$). However, the mean RTLs of 9q-short and 9q-long were not significantly different between cases and controls. When the case–control comparison was stratified by menopausal status, tobacco smoking status or physical activity in teens, we observed similar patterns of case–control differences across all subgroups of women.

RTL of chromosome 9p-short telomere and breast cancer risk

Table III shows the distributions of breast cancer patients and control subjects according to the RTL of 9p-short telomere. Using the 50th percentile value in controls as a cut point, women who had shorter 9p-short telomeres had significantly increased risk of breast cancer compared with women who had longer 9p-short telomeres [adjusted OR = 2.6; 95% confidence interval (CI) = 1.5–4.3] in the overall study population. When stratified by menopausal status, the ORs were 2.8 (95% CI = 1.3–6.2) and 2.4 (95% CI = 1.2–4.9) for pre- and postmenopausal women, respectively. When the RTL data were categorized into quartiles, a significant inverse dose–response relationship was observed ($P_{trend} < 0.001$), and the lowest-versus-highest quartile OR was 6.6 (95% CI = 2.8–15.9) for all women. The ORs were 6.2 (95% CI = 1.8–21.3) and 7.5 (95% CI = 2.1–26.8) for pre- and postmenopausal women, respectively.

RTL of chromosome 9p-long telomere and breast cancer risk

Table IV shows the distributions of breast cancer patients and control subjects according to the RTL of 9p-long telomere. Using the 50th value in controls as a cut point, women who had shorter 9p-long telomere had marginally significant increased risk of breast cancer compared with women who had longer 9p-long telomere (OR = 1.6, 95% CI = 1.0 to 2.7) in the overall study population. When the RTL

Table I. Distribution of characteristics of study population

Host factors	Cases (N = 140)	Controls (N = 159)	P-value
Age, mean (SD)	52.71 (10.61)	53.25 (9.92)	0.656
Race, *n* (%)			
Whites	104 (74.2)	118 (74.2)	
Blacks	28 (20.0)	34 (21.4)	
Others	8 (5.7)	7 (4.4)	0.850
Menopausal status, *n* (%)			
Premenopausal status	56 (42.8)	67 (42.4)	
Postmenopausal status	75 (57.3)	91 (57.6)	0.953
Tobacco smoking, *n* (%)			
Never	81 (60.0)	93 (58.9)	
Ever	54 (40.0)	65 (41.1)	0.843
Alcohol use, *n* (%)			
Never	15 (11.5)	12 (7.6)	
Ever	116 (88.6)	147 (92.5)	0.225
Physical activity in teens, *n* (%)			
No	55 (39.3)	31 (19.5)	
Yes[a]	85 (60.7)	128 (80.5)	**<0.001**
Physical activity last year, *n* (%)			
No	59 (42.1)	36 (22.6)	
Yes[a]	81 (57.9)	123 (77.4)	**<0.001**
Education, *n* (%)			
≤4 years college	78 (58.2)	82 (51.6)	
>4 years college	56 (41.8)	77 (48.4)	0.256
Family income, *n* (%)			
≤100K	46 (32.9)	56 (35.2)	
>100K	51 (36.4)	77 (48.4)	
Missing	43 (30.7)	26 (16.4)	**0.010**
Family history of cancer[b]			
No	50 (39.7)	73 (46.5)	
Yes	76 (60.3)	84 (53.5)	0.250
HRT, *n* (%)			
No	86 (65.7)	94 (59.1)	
Yes	45 (34.4)	65 (40.9)	0.254
BMI, mean (SD)	27.14 (6.30)	27.28 (6.64)	0.862
Total telomere length[c], mean (SD)	4.36 (0.99)	4.58 (1.01)	0.069

Bold numbers are statistically significant. BMI, body mass index; HRT, hormone replacement therapy.
[a]Defined as physical activity at least once a week for at least 20 min at a time that either made the woman sweat or increased heart rate.
[b]Defined as any cancer in the first degree blood relatives.
[c]The unit of total telomere length is fluorescent intensity units in million (MFIU).

Table II. Case–control comparison of mean chromosome 9 telomere lengths, by host factors

RTL (%)	Cases		Controls		P-value
	n	Mean (SD)	n	Mean (SD)	
All subjects					
9p-short TL	140	0.515 (0.12)	159	0.593 (0.12)	**<0.001**
9p-long TL	140	1.184 (0.18)	159	1.234 (0.17)	**0.017**
9q-short TL	140	0.512 (0.12)	159	0.525 (0.14)	0.403
9q-long TL	140	1.185 (0.19)	159	1.176 (0.20)	0.693
Premenopausal women					
9p-short TL	56	0.510 (0.13)	67	0.604 (0.11)	**<0.001**
9p-long TL	56	1.173 (0.18)	67	1.244 (0.17)	**0.040**
9q-short TL	56	0.531 (0.11)	67	0.529 (0.14)	0.957
9q-long TL	56	1.206 (0.20)	67	1.162 (0.20)	0.306
Postmenopausal women					
9p-short TL	75	0.516 (0.11)	91	0.582 (0.13)	**<0.001**
9p-long TL	75	1.189 (0.18)	91	1.226 (0.18)	0.267
9q-short TL	75	0.504 (0.13)	91	0.525 (0.14)	0.214
9q-long TL	75	1.173 (0.17)	91	1.185 (0.19)	0.486
Never smokers					
9p-short TL	81	0.514 (0.12)	93	0.604 (0.12)	**<0.001**
9p-long TL	81	1.191 (0.20)	93	1.233 (0.16)	0.132
9q-short TL	81	0.505 (0.13)	93	0.527 (0.14)	0.259
9q-long TL	81	1.190 (0.21)	93	1.178 (0.20)	0.711
Ever smokers					
9p-short TL	54	0.520 (0.11)	65	0.575 (0.13)	**0.014**
9p-long TL	54	1.180 (0.17)	65	1.238 (0.19)	0.077
9q-short TL	54	0.521 (0.12)	65	0.522 (0.14)	0.969
9q-long TL	54	1.182 (0.16)	65	1.177 (0.19)	0.862
Women who were not physically active in their teens					
9p-short TL	55	0.490 (0.12)	31	0.615 (0.13)	**<0.001**
9p-long TL	55	1.174 (0.18)	31	1.269 (0.15)	**0.011**
9q-short TL	55	0.513 (0.14)	31	0.530 (0.15)	0.601
9q-long TL	55	1.202 (0.21)	31	1.174 (0.19)	0.522
Women who were physically active in their teens					
9p-short TL	85	0.531 (0.12)	128	0.588 (0.13)	**<0.001**
9p-long TL	85	1.191 (0.18)	128	1.225 (0.18)	0.174
9q-short TL	85	0.512 (0.12)	128	0.524 (0.14)	0.494
9q-long TL	85	1.174 (0.18)	128	1.177 (0.20)	0.914

Bold numbers are statistically significant. RTL, relative telomere length.

data were categorized into quartiles, a significant inverse dose–response relationship was observed (P_{trend} = 0.020), and the lowest-versus-highest quartile OR was 2.2 (95% CI = 1.1–4.4) for all women. The OR was 3.0 (95% CI = 1.1–8.7) and 1.6 (95% CI = 0.6–4.3) for pre- and postmenopausal women, respectively.

Table V presents the joint effects of 9p-short and 9p-long RTL on breast cancer risk. The data suggested additive effects. However, no statistical significant interactions between 9p-short and 9p-long RTL were detected in all subjects (P = 0.108), premenopausal women (P = 0.113) or postmenopausal women (P = 0.415) when the interactions were formally tested in logistic models.

RTL of chromosome 9q-short and 9q-longtelomeres and breast cancer risk

Using the 50th percentile value in controls as a cut point, when women who had shorter 9q-short telomere were compared with women who had longer 9q-short telomere, the OR was 1.0 (95% CI = 0.6–1.7) for all women. When stratified by menopausal status, the ORs were 1.4 (95% CI = 0.7–2.7) and 0.8 (95% CI = 0.4–1.8) for pre- and postmenopausal women, respectively. When the data were categorized into quartiles, no significant dose–response relationship or a statistically significant difference by comparing the lowest to highest quartile was observed.

Similarly, when we used the 50th percentile value in controls as a cut point, women who had shorter 9q-long telomeres compared with longer 9q-long telomeres had an adjusted OR of 1.3 (95% CI = 0.8–2.0) overall. When stratified by menopausal status, the ORs were 1.6 (95% CI = 0.8–3.0) and 1.0 (95% CI = 0.5–2.1) for pre- and postmenopausal women, respectively. When the data were categorized into quartiles, no significant dose–response relationship or a statistically significant difference by comparing the lowest to highest quartile was observed.

Correlations of chromosome 9 telomere lengths and age

Among control subjects, we observed a weak inverse correlation between 9p-short telomere length and age [Pearson correlation coefficient (r) = −0.162, P = 0.042]. No significant correlations were seen between age and the other three chromosome 9 telomeres. There were significant correlations between the allelic 9p telomere lengths (r = 0.431, $P < 0.001$) and between the allelic 9q telomere lengths (r = 0.428, $P < 0.001$). There were no significant correlations between 9p telomeres and 9q telomere lengths, except for a weak correlation between 9p-short and 9q-short (r = 0.204, P = 0.010).

Among cases, there were no significant correlations between chromosome 9 telomere lengths and age. There were significant correlations between the allelic 9p telomeres (r = 0.525, $P < 0.001$) and between the allelic 9q telomeres (r = 0.326, $P < 0.001$). There were

Table III. Logistic regression analysis examining the association of chromosome 9p-short telomere length and breast cancer risk

9p-short RTL (%)	Cases/controls	OR[a] (95% CI)	*P*	OR[b] (95% CI)	*P*
All subjects					
≥Median (≥0.583)	41/81	1.00		1.00	
<Median (<0.583)	99/78	2.53 (1.56–4.12)	**<0.001**	2.58 (1.54–4.33)	**<0.001**
Long (≥0.680)	10/41	1.00		1.00	
Short (<0.680)	130/118	5.08 (2.35–10.96)	**<0.001**	4.43 (1.98–9.88)	**<0.001**
By quartiles					
Q4 (≥0.680)	10/41	1.00		1.00	
Q3 (0.583–0.680)	31/40	3.43 (1.44–8.19)		3.00 (1.20–7.45)	
Q2 (0.494–0.583)	35/40	3.93 (1.66–9.29)		3.87 (1.58–9.49)	
Q1 (≤0.494)	64/38	7.51 (3.25–17.37)	**<0.001**[c]	6.62 (2.75–15.94)	**<0.001**[c]
Premenopausal women					
≥Median (≥0.583)	17/38	1.00		1.00	
<Median (<0.583)	39/29	3.00 (1.42–6.35)	**0.004**	2.80 (1.28–6.16)	**0.010**
Long (≥0.680)	5/18	1.00		1.00	
Short (<0.680)	51/49	3.91 (1.34–11.45)	**0.013**	3.62 (1.18–11.08)	**0.024**
By quartiles					
Q4 (≥0.680)	5/18	1.00		1.00	
Q3 (0.583–0.680)	12/20	2.29 (0.67–7.85)		2.16 (0.60–7.83)	
Q2 (0.494–0.583)	11/14	2.90 (0.81–10.35)		2.87 (0.76–10.81)	
Q1 (≤0.494)	28/15	6.99 (2.14–22.83)	**<0.001**[c]	6.16 (1.78–21.32)	**0.002**[c]
Postmenopausal women					
≥Median (≥0.583)	22/42	1.00		1.00	
<Median (<0.583)	53/47	2.08 (1.09–3.99)	**0.027**	2.41 (1.20–4.87)	**0.014**
Long (≥0.680)	4/22	1.00		1.00	
Short (<0.680)	71/67	5.89 (1.91–18.14)	**0.002**	5.66 (1.75–18.28)	**0.004**
By quartiles					
Q4 (≥0.680)	4/22	1.00		1.00	
Q3 (0.583–0.680)	18/20	5.21 (1.49–18.18)		4.32 (1.15–16.26)	
Q2 (0.494–0.583)	21/26	4.60 (1.36–15.49)		5.13 (1.45–18.13)	
Q1 (≤0.494)	32/23	8.24 (2.46–27.66)	**0.002**[c]	7.53 (2.11–26.79)	**0.003**[c]

Bold numbers are statistically significant.
[a]OR adjusted for age as continuous and race.
[b]OR adjusted for age as continuous, race, smoking status (never/ever), alcohol use (never/ever), education, family history of cancer (no/yes), hormone replacement therapy (no/yes), history of pregnancy (no/yes), menopausal status (when appropriate) and physical activity in teens (no/yes).
[c]*P* for trend.

statistically significant but weak correlations between 9p-short and 9q-short ($r = 0.256$, $P = 0.002$) and between 9p-long and 9q-long ($r = 0.186$, $P = 0.028$).

Discussion

Our results showed that, after adjustment for known breast cancer risk factors, shorter telomeres on chromosome 9p-short (allelic shorter version) were strongly associated with an increased risk of breast cancer. This finding supports our hypothesis that individuals who possess short telomeres on chromosome 9 are at increased risk of breast cancer. Our results are corroborated by previous reports showing that telomere dysfunction is involved in the development of breast cancer (6,7). For example, marked shortening of chromosome telomeres were reported in grade II and III breast cancers (35). Very short telomeres are also frequent genetic alterations in premalignant lesions (6,36), suggesting that telomere dysfunction is an early event in breast carcinogenesis.

A study of genetically engineered mice provided evidence that the average telomere length in a cell may be less important for cancer risk than short telomeres on specific chromosome arms because chromosome fusions associated with telomere dysfunction occur preferentially on chromosome arms with the shortest telomeres (37). In humans, there are 23 pairs of chromosomes and 92 telomeres, and chromosome-specific telomere lengths are highly polymorphic between chromosomal arms (38–40). One potential implication of this chromosome arm-specific telomere length variation is that chromosome arms bearing the shortest telomeres may predispose to the chromosome aberrations and therefore have an impact on the evolution of tumors. This concept is supported by several studies demonstrating that chromosome arms with the shortest telomeres were more often found in the telomere fusions leading to chromosome instability (37,41). Our observation that shortest 9p telomere (shorter 9p-short telomeres) was strongly associated with breast cancer risk supports this concept. To the best of our knowledge, our study is the first to report that shorter telomere on chromosome 9p-short is strongly associated with breast cancer risk. If confirmed by other studies, chromosome 9p telomere length, in combination with other risk biomarkers, could potentially improve breast cancer risk assessment for individual women in general population. Based on these promising findings, it is reasonable to anticipate that lengths of other arm-specific telomeres might be associated with breast cancer risk. Thus, future studies should aim to identify such telomeres.

Our results also indicated that telomere lengths on chromosome 9q were not associated with breast cancer risk. We observed no significant correlations between 9p and 9q telomere lengths in controls, except a weak correlation between 9p-short and 9q-short, suggesting telomere lengths on 9p or 9q are independent events. It is well documented that frequent chromosomal abnormalities in early stage breast cancers only involves a handful of chromosome arms, including gains of 1q, 8q, 17q and 20q and losses of 8p, 9p, 16q and 17p (5,8,9). Therefore, it is not surprising to find that chromosome 9q telomeres were not associated with breast cancer risk in our study because chromosome 9q aberration is not a common abnormality in breast tumors. In this study, we also found that there was no significant difference in mean overall (cell total) telomere length between cases and controls (Table I) (31). It would be expected that overall telomere length would not be informative due to the inclusion of large number of 'irrelevant' telomeres in the measurement. This may in part explain

Table IV. Logistic regression analysis examining the association of chromosome 9p-long telomere length and breast cancer risk

9p-long RTL (%)	Cases/controls	OR[a] (95% CI)	*P*	OR[b] (95% CI)	*P*
All subjects					
≥Median (≥1.211)	54/81	1.00		1.00	
<Median (<1.211)	86/78	1.65 (1.03–2.64)	**0.037**	1.63 (0.99–2.67)	0.056
Long (≥1.343)	24/40	1.00		1.00	
Short (<1.343)	116/119	1.56 (0.89–2.796)	0.118	1.56 (0.83–2.79)	0.170
By quartiles					
Q4 (≥1.343)	24/40	1.00		1.00	
Q3 (1.211–1.343)	30/41	1.18 (0.59–2.36)		1.12 (0.54–2.36)	
Q2 (1.122–1.211)	35/39	1.47 (0.73–2.94)		1.27 (0.60–2.66)	
Q1 (≤1.122)	51/39	2.11 (1.09–4.07)	**0.018**[c]	2.19 (1.09–4.39)	**0.020**[c]
Premenopausal women					
≥Median (≥1.211)	21/36	1.00		1.00	
<Median (<1.211)	35/31	1.97 (0.95–4.12)	0.070	1.84 (0.84–4.00)	0.126
Long (≥1.343)	10/20	1.00		1.00	
Short (<1.343)	46/47	2.00 (0.84–4.77)	0.117	1.84 (0.74–4.60)	0.192
By quartiles					
Q4 (≥1.343)	10/20	1.00		1.00	
Q3 (1.211–1.343)	11/16	1.41 (0.48–4.19)		1.32 (0.42–4.19)	
Q2 (1.122–1.211)	12/16	1.52 (0.52–4.47)		1.18 (0.36–3.81)	
Q1 (≤1.122)	23/15	3.18 (1.16–8.74)	**0.025**[c]	3.03 (1.06–8.70)	**0.042**[c]
Postmenopausal women					
≥Median (≥1.211)	31/44	1.00		1.00	
<Median (<1.211)	44/47	1.35 (0.72–2.52)	0.345	1.45 (0.75–2.80)	0.275
Long (≥1.343)	13/20	1.00		1.00	
Short (<1.343)	62/71	1.36 (0.62–2.96)	0.446	1.26 (0.55–2.88)	0.586
By quartiles					
Q4 (≥1.343)	13/20	1.00		1.00	
Q3 (1.211–1.343)	18/24	1.15 (0.45–2.93)		0.96 (0.35–2.60)	
Q2 (1.122–1.211)	19/23	1.29 (0.50–3.31)		1.17 (0.43–3.20)	
Q1 (≤1.122)	25/24	1.61 (0.66–3.96)	0.270[c]	1.64 (0.63–4.26)	0.233[c]

Bold numbers are statistically significant.
[a]OR adjusted for age as continuous and race.
[b]OR adjusted for age as continuous, race, smoking status (never/ever), alcohol use (never/ever), education, family history of cancer (no/yes), hormone replacement therapy (no/yes), history of pregnancy (no/yes), menopausal status (when appropriate) and physical activity in teens (no/yes).
[c]*P* for trend.

Table V. Joint effect of chromosome 9p-short and 9p-long telomere length on breast cancer risk

9p-short	9p-long	Cases/controls	OR (95% CI)
All subjects			
Long[a]	Long[a]	30/53	1.00
Short	Long	24/28	2.71 (1.45–5.04)
Long	Short	11/28	1.73 (0.85–3.54)
Short	Short	75/50	3.97 (1.71–9.19)
Premenopausal women			
Long	Long	13/25	1.00
Short	Long	8/11	3.05 (1.20–7.78)
Long	Short	4/13	2.44 (0.73–8.24)
Short	Short	31/18	5.49 (1.46–20.7)
Postmenopausal women			
Long	Long	16/27	1.00
Short	Long	15/17	2.44 (1.04–5.71)
Long	Short	6/15	1.39 (0.56–3.45)
Short	Short	38/32	3.25 (1.08–9.82)

ORs were adjusted for age as continuous, race, smoking status (never/ever), alcohol use (never/ever), education, family history of cancer (no/yes), hormone replacement therapy (no/yes), history of pregnancy (no/yes), menopausal status (when appropriate) and physical activity in teens (no/yes). No significant interactions between 9p-short and 9p-long were detected.
[a]Chromosome 9p-short and 9p-long RTL were dichotomized by the median value in controls.

the null findings by three recent studies that examined the association of overall (cell total) telomere length in blood leukocytes and breast cancer risk (32–34).

The case–control differences reported here are unlikely to be ascribed to assay bias because the samples were processed and measured blinded to case–control status. Measurements of four chromosome 9-arm telomere lengths were made simultaneously from the same cells captured by the fluorescent imaging system. While there were significant case–control differences for chromosome 9p telomere lengths, the mean telomere lengths of chromosome 9q and cell total were very similar between cases and controls. During the study, a systematic quality control plan was implemented to ensure the consistent efficiency of fluorescent *in situ* hybridization (the coefficient of variation of the total telomere lengths among 20 repeats of the control cell = 12.4%). Co-hybridization of a FITC-labeled chromosome 9-specific probe (green) was used as an internal control for hybridization efficiency, and only slides that showed bright green chromosome 9 signals were accepted. The samples that failed to meet these quality control standards were rejected and repeated (3% of the samples). Additionally, relative telomere lengths of chromosome 9 arms were defined as the ratio of chromosome 9 telomere lengths to the total telomere length, thus minimizing the assay variation between the individual samples. Bias in telomere length measurement is thus unlikely.

Given that this is a case–control study, a theoretical concern is that telomere length in leukocytes 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 (31–34), suggesting there is no significant shortening of blood leukocyte telomere length associated with having breast cancer. Although previous studies (42,43) 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 results.

Recall bias might influence information about self-reported breast cancer risk factors in a case–control study where the data were collected after the diagnosis of cancer. We compared chromosome 9 telomere lengths in control subjects by numerous variables from the questionnaire and did not find any significant differences in the mean chromosome 9 telomere lengths between subgroups defined by race, age, smoking status, alcohol drinking, menopausal status, physical activity in the teens, hormone replacement therapy, history of pregnancy, family history of cancer, education and income (data not shown). Cases and controls were closely matched by age, and age was included as continuous variable in all the logistic models for adjustment. Thus, age would not confound the telomere results.

In conclusion, our data provide the first evidence that short telomere on chromosome 9p is strongly associated with breast cancer risk. If confirmed in future studies, chromosome 9p telomere length could be incorporated into the current prediction models to significantly enhance breast cancer risk prediction. Better risk assessment would improve the efficiency of both population-based preventive programs, such as screening mammography, as well as individual-based preventive strategies such as chemoprevention by targeting women who are at the greatest risk for breast cancer.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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