

Telomere Length in Prospective and Retrospective Cancer Case-Control Studies

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Abstract

Previous studies have reported that shorter mean telomere length in lymphocytes was associated with increased susceptibility to common diseases of aging, and may be predictive of cancer risk. However, most analyses have examined retrospectively collected case-control studies. Mean telomere length was measured using high-throughput quantitative real-time PCR. Blood for DNA extraction was collected after cancer diagnosis in the East Anglian SEARCH Breast (2,243 cases and 2,181 controls) and SEARCH Colorectal (2,249 cases and 2,161 controls) studies. Prospective case-control studies were conducted for breast cancer (199 cases) and colorectal cancer (185 cases), nested within the EPIC-Norfolk cohort. Blood was collected at least 6 months prior to diagnosis, and was matched to DNA from two cancer-free controls per case. In the retrospective SEARCH studies, the age-adjusted odds ratios for shortest (Q4) versus longest (Q1) quartile of mean telomere length was 15.5 [95% confidence intervals (CI), 11.6–20.8; *p*-het = 5.7×10^{-75}], with a “per quartile” *P*-trend = 2.1×10^{-80} for breast cancer; and 2.14 (95% CI, 1.77–2.59; *p*-het = 7.3×10^{-15}), with a per quartile *P*-trend = 1.8×10^{-13} for colorectal cancer. In the prospective EPIC study, the comparable odds ratios (Q4 versus Q1) were 1.58 (95% CI, 0.75–3.31; *p*-het = 0.23) for breast cancer and 1.13 (95% CI, 0.54–2.36; *p*-het = 0.75) for colorectal cancer risk. Mean telomere length was shorter in retrospectively collected cases than in controls but the equivalent association was markedly weaker in the prospective studies. This suggests that telomere shortening largely occurs after diagnosis, and therefore, might not be of value in cancer prediction.

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Introduction

Human chromosomes are capped and stabilized by telomeres, which are predominantly formed from several thousand (TTAGGG)_n repeats (1–3) and are heterogeneous in length, varying between chromosomes and individuals (4–8). Telomeres prevent chromosome ends from being recognized as damaged DNA in need of double-strand break repair and, as a result, protect against chromosome-chromosome fusions and rearrangements, helping maintain genomic integrity (9–12).

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Telomeric DNA is inefficiently copied. This is referred to as “the end replication problem” (1, 13, 14) and leads to a progressive loss in mean telomere length estimated at 15 to 60 bp per year (15, 16), with this rate increasing over the age of 50 years (5, 17). There are reports that telomere attrition may be accelerated by environmental cofactors such as smoking (18–20), oxidative stress (21–23), poor physical health (24–26), and obesity (18). Thus, mean telomere length has been suggested as a measure of the “biological age” of both the cell and the organism (27, 28).

Previous experimental studies have indicated that individuals with relatively short mean telomere lengths might have an increased risk of mortality from multiple diseases (20, 29–38). In particular, five studies have examined the associations between breast cancer risk and mean telomere length (35–39). The first, by Shen and colleagues (35), reported an increased risk of breast cancer of borderline statistical significance associated with shorter telomere length (287 cases and 350 sister controls); odds ratios (OR) [shortest Q4 versus longest Q1 (referent)] = 1.55 [95% confidence intervals (CI), 0.88–2.73; *P* = 0.14]. A second, larger study from the same group (36) reported a significant association with breast cancer risk in women under 50 years of age: OR (Q4 versus Q1) = 1.78 (95% CI, 1.15–2.76; *P* = 0.01). No association was seen between mean telomere length and breast cancer in women 50 years of age or older (36). In both of these studies,

blood samples for DNA extraction from the case individuals were collected retrospectively—after cancer diagnosis.

Zheng and colleagues (37) investigated mean telomere length and breast cancer risk in two small, retrospectively collected studies. The combined OR for both studies (292 cases and 335 controls) associated with a telomere length “below” versus “above” the median length was 1.23 (95% CI, 0.89–1.71; $P = 0.13$).

Another study (38) looked directly at mean telomere length and both cancer risk and survival. In contrast with the other articles, Svenson and colleagues reported an increased risk of breast cancer with increasing telomere length: [longest Q1 versus shortest Q4 (referent)] OR = 5.17 (95% CI, 3.09–8.64; $P = 0.001$), and showed consistently longer telomeres in cases than in controls for all age groups.

Recently, De Vivo and colleagues (39) published the largest and only prospectively designed breast cancer case-control study to date, with blood extracted from individuals prior to disease diagnosis or development, in 1,122 postmenopausal women and 1,147 matched controls. Using real-time PCR to measure mean telomere length, they estimated an OR for telomere length below versus above the median length of 1.23 (95% CI, 0.94–1.60; $P = 0.20$).

Thus far, there have been two studies published looking at the relationship between mean telomere length and colorectal cancer (40, 41). These prospective, nested case-control comparisons in men (191 cases and 306 controls) and women (134 cases and 357 controls) showed no association between telomere length and disease status.

Here, we have measured mean telomere length in prospectively and retrospectively collected breast and colorectal cancer case-control series to more precisely evaluate the association between telomere length and cancer status, and to test whether mean telomere length has predictive value in cancer susceptibility.

Materials and Methods

Study populations

Retrospective SEARCH breast cancer case-control study. The SEARCH Breast study (42) is an ongoing population-based study, recruiting cases ascertained through the Eastern Cancer Registration and Information Centre (<http://www.ecric.org.uk/>), a population-based cancer registry covering the counties of Cambridgeshire, Norfolk, Suffolk, Bedfordshire, Hertfordshire, and Essex. Women aged under 70 y who were diagnosed from 1996 onwards (incident cases; median age, 55 y) were eligible for inclusion. Approximately 64% of eligible patients were enrolled in the study. Study participants were asked to provide a 20 mL blood sample for DNA analysis and to complete a comprehensive epidemiologic questionnaire. Eligible patients who did not take part in the study were similar to participants except the proportion of clinical stage III/IV cases was somewhat higher among nonparticipants.

For this report, the most recently accrued, extracted, and normalized DNA samples were used, consisting of 2,243 incident cases, diagnosed between 2004 and 2007, and 2,181 controls. The majority ($n = 1,524$) of the controls were collected

as cancer history-free control participants in the SEARCH study, recruited over the period 2003 to 2007. The remainder ($n = 657$) were cancer-free women selected from the Norfolk component of the European Prospective Investigation into Cancer (EPIC). Controls were broadly similar in age to the cases (median age of SEARCH controls, 53 y; median age of EPIC controls, 54 y) and SEARCH controls were additionally matched to the area of residence and the age group frequencies of the SEARCH cases. Additional study characteristics are presented in Supplementary Table S1A.

Retrospective SEARCH colorectal cancer case-control study. Cases were selected from the SEARCH Colorectal Study and ascertained through the Eastern Cancer Registration and Information Centre as described for the breast cancer study (above). Eligible patients for recruitment were diagnosed with invasive colorectal cancer or anal adenocarcinoma, and were ages 18 to 69 y at diagnosis (between March 2001 and February 2004). Approximately 63% of eligible patients were enrolled in the study. Eligible patients who did not take part in the study were similar to participants except that the proportion of clinical stage III/IV cases was somewhat higher among nonparticipants.

Two thousand two-hundred and forty-nine SEARCH colorectal cases and 2,161 controls were analyzed. All controls were recruited as cancer history-free control participants in the SEARCH study, and were enrolled from 2002 to 2005. Controls were frequency-matched with respect to gender, age in 5-y bands, and the area of residence of the cases. Additional study characteristics are presented in Supplementary Table S1A.

Prospective EPIC breast and colorectal nested case-control studies. Cases and controls were sampled from the Norfolk cohort of EPIC (<http://www.srl.cam.ac.uk/epic/>). EPIC is an ongoing prospective study of diet and cancer being carried out in nine European countries. The EPIC-Norfolk cohort comprises over 30,000 individuals, ages 45 to 75 at recruitment, resident in Norfolk, East Anglia, and recruited from general practice registers between 1993 and 1997. It is an ethnically homogeneous population with >99% reported as white European. A total of 25,639 participants completed an initial health examination. In January 1998, the cohort was invited for a second health examination, and 15,786 people took part in this second phase. All participants gave informed consent and were matched to the East Anglian Cancer Registry and the U.K. Office of National Statistics Register. These provided notification of all cancer registrations, deaths, and emigrations for the entire cohort, thus, loss to follow-up was <0.1%.

Eligible cases for this analysis were study participants who were cancer-free at baseline assessment and who were subsequently diagnosed with incident breast or colorectal cancer at least 6 mo after blood draw and up to the end of December 2003. Full data was available for telomere length analysis on 199 and 185 cases of incident breast and colorectal cancer, respectively. Eligible controls were study participants who remained free of cancer during this time. Two controls were matched to each case by gender, age (within 1 y), and date of blood draw (within 3 mo). In total, 384 cases of incident breast or colorectal cancer and 826 matched

control participants were available for analysis. The mean time from blood collection to date of diagnosis was 3.3 y (range, 1.4–4.6 y). Additional study characteristics are presented in Supplementary Table S1B. Ethical approval was obtained from the Norwich District Health Authority Ethics Committee for all the studies used.

Real-time PCR method

Relative mean telomere length was ascertained by SYBR Green Real-time PCR measurement of the ratio of telomere repeat units (TEL) to a single-copy gene (CON) (20, 43). The TEL assay amplifies a 78 bp telomeric repeat unit and fluorescence detected was proportional to the number of telomeric repeats in the genome available for primer binding, and thus, the mean telomere length in the cells from which the DNA was extracted. The CON assay amplifies a segment of the single-copy human β -globin gene sequence and was used to correct for sample-to-sample variation in template DNA added to the reaction.

For each assay, the fractional PCR cycle at which each reaction crossed a predefined fluorescence threshold was determined (“Ct value”). The amount of the starting template was expected to be proportional to 2^{-Ct} . To correct for variation in genomic DNA concentration, the CON Ct value was subtracted from the TEL Ct value (ΔCt). The relative “telomere copy number” per genome for each sample should then be proportional to $2^{-\Delta Ct}$. Ten nanograms of genomic DNA, dried down in a 384-well plate format, was resuspended in 10 μ L of either the CON or TEL PCR reaction mix for 2 h at 4°C prior to thermal cycling.

The PCR primer sequences for both PCR reactions have been previously published (20). Each 10 μ L CON reaction contained 300 and 700 nmol/L of the forward and reverse primers (Sigma Aldrich), respectively, and 5 μ L of 2 \times Power SYBR Green PCR Master Mix (Applied Biosystems). The PCR profile was an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 28 s. Each 10 μ L TEL reaction contained 100 and 900 nmol/L of the forward and reverse primers (Sigma Aldrich), respectively, 5 μ L of 2 \times Power SYBR Green PCR Master Mix (Applied Biosystems), and 0.3 μ L of DMSO PCR Reagent (Sigma Aldrich). The PCR profile was an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 54°C for 2 min.

All reactions were performed using an ABI7900 thermal cycler, in 384-well format. Each plate was composed of equally interdigitated cases and controls, and included negative control wells (containing no DNA). Thirty-two percent ($n = 382$) of the prospective EPIC study and 12% ($n = 526$) of the retrospective SEARCH studies were run in duplicate, with repeated samples assayed in a secondary run during the experiment, using a separately prepared mix of PCR reagents.

A standard plate of “test” samples was additionally assayed. This plate consisted of 94 high-yield DNA samples and was assayed in each PCR batch performed, i.e., in triplicate, as a method of interexperiment quality control. Failed PCR reactions were not repeated (as defined below).

Statistical methods

Analyses were based primarily on the ΔCt variable, rather than the relative copy number ($2^{-\Delta Ct}$), as the copy number data was positively skewed, whereas the ΔCt values fit a normal distribution well, making it unnecessary to transform the raw ΔCt data twice. The interexperimental quality control comparisons of repeated samples were assessed using Pearson correlation calculations. The intraexperimental comparison of standard test plates, for assurance of study-to-study quality control, was assessed using Spearman rank correlations. For all analyses, “outlier” samples were removed if the CON PCR Ct value was >2 SD away from the mean, and these reactions were considered “fails.”

The association between ΔCt and age at blood draw was evaluated using unconditional linear regression, adjusting for study, gender, and individual 384-well sample plate.

In each case-control study, we used logistic regression to assess the association between mean telomere length and cancer status (breast or colorectal). Subjects were categorized into quartiles for telomere length, the boundaries of which were defined by the distribution of ΔCt in the control sample population of each study. Adjustments are detailed in Tables 1 and 2. All analyses were performed using Intercooled Stata 10.1 statistical package (Stata).

Results

Quality control

As a measure of assay quality assurance across the three case-control series, the correlation between repeated measurements of the same samples, assayed in separate PCR batches, was calculated. This was ≥ 0.83 for the ΔCt values (≥ 0.92 for the CON PCR, ≥ 0.93 for the TEL PCR) in each study. More than 97% of the samples attempted gave results. The Spearman rank order correlations across the triplicate test plate assays were ≥ 0.71 for the ΔCt values (≥ 0.72 for the CON PCR, ≥ 0.87 for the TEL PCR). As a further validation of the assay used, the known association of mean telomere length with age in the unaffected controls in the sample sets studied ($n = 5,256$) was examined. As expected, there was a significant decrease in mean telomere length with age (after adjustment for study, gender, and 384-well plate); increase in ΔCt “per annum” was 0.0045 (95% CI, 0.003–0.006; P -trend = 1.5×10^{-9}).

Mean telomere length and breast cancer

The estimated ORs by quartile of ΔCt , adjusted for age and plate, in the retrospective and prospective breast cancer studies are shown in Table 1. A stronger association was observed in the retrospective study compared with the prospective study. In the retrospective study, there was a trend across decreasing quartiles of telomere length, with a “per quartile”, plate and age-adjusted OR of 2.56 (95% CI, 2.32–5.23; P -trend = 2.1×10^{-80}). The OR for the shortest (Q4) versus the longest (Q1) quartile of ΔCt was 15.5 (95% CI, 11.6–20.8; p -het = 5.7×10^{-75}). Although cases were matched to controls from two different sources in this study, there was little difference in the effect size between those matched to SEARCH

controls (OR per quartile = 2.44; 95% CI, 2.21–2.70; *P*-trend = 8.5×10^{-69}) and those matched to controls ascertained through EPIC-Norfolk (2.18; 95% CI, 1.90–2.51; *P*-trend = 2.5×10^{-28}). There was no significant difference between the effect sizes in subjects under 50 years of age or 50 years and older, a division approximating premenopausal and postmenopausal status (data not shown).

The distributions of Δ Ct in cases and controls (for each study) are further illustrated in Supplementary Fig. S1. In all four panels, the case distribution is skewed to the right of the control distribution, but this is most marked in the retrospective breast cancer study (bottom left), in which almost half of the cases (1,013 of 2,243 or 45%) lie in the shortest quartile for length, Q4.

In the prospective breast cancer study, the association with Δ Ct was in the same direction as the retrospective breast cancer study, but was of a much smaller magnitude and not statistically significant; plate and age-adjusted OR per quartile was 1.18 (95% CI, 0.93–1.50; *P*-trend = 0.18) and OR (Q4 versus Q1) was 1.58 (0.75–3.31; *p*-het = 0.23).

Mean telomere length and colorectal cancer

The colorectal cancer case-control results followed a similar pattern to those of the breast cancer studies, albeit with smaller point estimates of risk (Table 2). In the retrospective colorectal study, the plate and age-adjusted OR per quartile was 1.25 (95% CI, 1.18–1.33; *P*-trend = 1.18×10^{-13}) and OR (Q4 versus Q1) was 2.14 (95% CI, 1.77–2.59; *p*-het = 7.3×10^{-15}). There was no evidence for an association in the prospective colorectal cancer study. The plate and age-adjusted OR per quartile was 1.03 (95% CI, 0.81–1.30; *P*-trend = 0.82) and OR (Q4 versus Q1) was 1.13 (0.54–2.36; *p*-het = 0.75).

Discussion

We have adapted a quantitative PCR assay (20, 43) to evaluate telomere length in lymphocytes in large-scale epidemiologic studies. We have shown that this methodology is reproducible in multiply-repeated assays, and shows the expected shortening of telomere length known to occur with increasing age.

We found a strong association between shorter telomere length and breast cancer status in the retrospective study, with an OR of ~ 15 for women in the bottom quartile of telomere length compared with the top quartile. The prospective breast cancer study showed some evidence of an association in the same direction, but the effect was not statistically significant. Thus, the hypothesis that mean telomere length in lymphocytes is a predictor of cancer risk seems to have been overstated. All but one of the previously published studies looking at associations between breast cancer and mean telomere length showed an association in the same direction as we report here. However, most of these studies have effect sizes of a similar magnitude to that seen in the prospective study, even though these were predominantly retrospectively collected. The only other truly prospective study of breast cancer risk and mean telomere length (39) gave a similar risk estimate to ours: OR (below versus above the median length) = 1.23 (95% CI, 0.94–1.60; *P*-trend = 0.20) versus OR = 1.52 (95% CI, 0.90–2.58; *p*-het = 0.12), respectively.

It is possible that the results from the prospective studies have underestimated the true size of any association, perhaps due to the comparatively small size of these studies, or simply random measurement error. It is unlikely that

Table 1. Telomere length in prospective and retrospective breast cancer case-control studies

Relative telomere length (adjusted for covariates)*	Breast cancer OR (95% CI), <i>p</i> -het	
	Prospective EPIC	Retrospective SEARCH
	199 cases and 420 controls	2,243 cases and 2,181 controls
Q1 longest	1.00 referent	1.00 referent
Q2	1.04 (0.57–1.89), 0.91	2.03 (1.63–2.53), 2.3×10^{-10}
Q3	1.54 (0.80–2.98), 0.20	4.59 (3.51–6.00), 6.4×10^{-29}
Q4 shortest	1.58 (0.75–3.31), 0.23	15.5 (11.6–20.8), 5.7×10^{-75}
Per quartile	1.18 (0.93–1.50)	2.56 (2.32–2.82)
	<i>P</i> -trend = 0.18	<i>P</i> -trend = 2.1×10^{-80}
Median split†	1.52 (0.90–2.58)	4.29 (3.52–5.23)
	<i>p</i> -het = 0.12	<i>p</i> -het = 9.2×10^{-47}

NOTE: Subjects were categorized into quartiles of telomere length, defined by the continuous distribution of Δ Ct in the respective study's control sample population. The Q1 referent quartile group had the longest mean telomere length and the Q4 quartile group had the shortest.

*The prospective EPIC study was matched for age and gender, and analysis adjusted for study plate. The retrospective SEARCH study analysis was adjusted for study plate and age.

†The median split refers to the division of the samples into those "above" vs. "below" the median length, i.e., samples in Q1 and Q2 (referent) vs. Q3 and Q4.

Table 2. Telomere length in prospective and retrospective colorectal cancer case-control studies

Relative telomere length (adjusted for covariates)*	Colorectal cancer OR (95% CI), p-het	
	Prospective EPIC	Retrospective SEARCH
	185 cases and 406 controls	2,249 cases and 2,161 controls
Q1 longest	1.00 referent	1.00 referent
Q2	1.09 (0.56–2.10), 0.81	1.51 (1.24–1.84), 4.7×10^{-5}
Q3	0.71 (0.34–1.47), 0.35	1.47 (1.21–1.79), 1.3×10^{-4}
Q4 shortest	1.13 (0.54–2.36), 0.75	2.14 (1.77–2.59), 7.3×10^{-15}
Per quartile	1.03 (0.81–1.30) <i>P</i> -trend = 0.82	1.25 (1.18–1.33) <i>P</i> -trend = 1.8×10^{-13}
Median split†	0.85 (0.51–1.40) p-het = 0.52	1.43 (1.25–1.63) p-het = 1.4×10^{-7}

NOTE: Subjects were categorized as detailed in the legend of Table 1.

*The prospective EPIC study was matched and analyzed as in Table 1. The retrospective SEARCH study analysis was adjusted for study plate, gender, and age.

†The median split is defined in the Table 1 legend.

study size led to us missing a true association of the magnitude of the SEARCH retrospective study. In our breast cancer studies, there was very little overlap in the confidence intervals of the prospective and retrospective studies, and the work published by De Vivo and colleagues (39) shows similar point estimates and confidence intervals using a study twice the size of ours.

The effect we have seen in our substantial retrospective breast cancer study is much larger than previous reports. The level of statistical significance may, in part, be due to the increased study size, but the large OR raises the possibility that this association may be artifactual, due to differences in DNA collection, storage, quality or other biases that retrospectively collected studies are prone to. This real-time PCR assay is very dependent on uniform DNA quality. However, we found little difference in mean telomere length between controls obtained from different sources. Furthermore, there was no correlation between DNA extraction yield, which we hypothesized might be related to DNA quality, and Δ Ct value in the retrospective samples. No plate bias was apparent, when using the original, whole study Δ Ct boundaries for quartile calculation, or when the quartiles of Δ Ct were recalculated on a plate-by-plate basis (Supplementary Fig. S2). However, what is apparent from the Δ Ct values along the X axes in Supplementary Fig. S1 is that the ranges were different from experiment to experiment (n.b., the two prospective studies were assayed together in a single experiment). It should also be noted that the mean telomere lengths generated from experiment to experiment are relative to that experiment, rather than absolute. They could vary according to PCR efficacy and reagent batch, but these factors have little effect on rank or quartile assignment.

The risk of colorectal cancer was not significantly affected by mean telomere length in our prospective study of 185 cases and 406 controls (Table 2). This agrees with the other similarly sized studies published to date (40, 41), looking

directly at colorectal cancer risk and mean telomere length. In the retrospective colorectal study, we found a significant association between mean telomere length and cancer status, but the effect was much weaker than that seen in the retrospective breast study; per quartile OR = 1.25 (95% CI, 1.18–1.33; p-het = 1.8×10^{-13}). It is possible that in a larger prospective colorectal cancer study, we may see a significant association, as there is a larger confidence interval overlap between our colorectal studies than that seen between the breast cancer studies, but we would not anticipate a large effect size.

The mean telomere length differences observed in these retrospective studies could be an effect of cancer treatment. Both radiotherapy and chemotherapy could cause DNA damage, but it is unclear whether this could have a significant effect on lymphocytes or on particular lymphocyte subpopulations. We found no evidence for any difference in Δ Ct in the retrospective cases according to whether the recorded treatment involved chemotherapy, hormone therapy, or radiotherapy (Supplementary Table S2). However, as we were not able to evaluate the combinations and duration of treatment in detail, it remains possible that telomere attrition is a response to a particular aspect of treatment or treatment regime. Alternatively, changes in telomere length may occur systemically during disease development. If so, mean telomere length could be an important screening marker, although not causally related to risk.

It could be that a more relevant measure of telomere length attenuation, with respect to cancer risk, is at the level of the individual chromosome, rather than the cellular mean. It may also be important to evaluate mean telomere length in solid tumor DNA and it would be interesting to investigate the relationship between this measurement and cancer development and treatment. These experiments are, however, beyond the scope of this particular study.

We believe we have excluded most artifactual bias as the cause for the difference between the results of our prospectively and retrospectively collected studies, and so our data suggests that the majority of telomere attrition occurs after cancer diagnosis rather than before or during cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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