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Short Communication**Telomere Length in the Colon Declines with Age: a Relation to Colorectal Cancer?**

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Abstract

Telomeres shorten with age, which may be linked to genomic instability and an increased risk of cancer. To explore this association, we analyzed telomere length in normal colorectal tissue of individuals at different ages using quantitative-fluorescence *in situ* hybridization (Q-FISH) and quantitative-PCR (Q-PCR). Using Q-FISH, we also examined the histologically normal epithelium adjacent to, or distant from, colon adenomas and cancers, in addition to the neoplasms. Q-FISH and Q-PCR showed that telomere length was inversely associated with age until ~ages 60 to 70; surprisingly, beyond this age, telomere length was positively associated with age. This association was found exclusively in epithelial, and not in stromal, cells. Peripheral blood lymphocytes showed an inverse association between

telomere length and age, but without any apparent increase in telomere length in the oldest individuals. Telomere length in larger adenoma lesions (>2 cm) was significantly shorter than in normal adjacent ($P = 0.004$) or normal distant ($P = 0.05$) tissue from the same individuals. However, telomere length in histologically normal epithelium adjacent to cancers or in adenomas <2 cm was not statistically different from that of the normal distant mucosa or from normal controls, evidence that a telomere-shortening field effect was not present. We suggest that the positive association between telomere length and age in the oldest patients is a consequence of selective survival of elderly patients with long colonocyte telomeres. (Cancer Epidemiol Biomarkers Prev 2006;15(3):573-7)

Introduction

Telomeres are repetitive DNA sequences (TTAGGG) found at the end of each chromosome. They provide stability and protect chromosomes from end-to-end fusions, degradation, and recombination (1). Telomere attrition can expose chromosome ends, activating cell cycle checkpoints and cellular senescence (2), and promoting cycles of bridge-breakage-fusion (3). The enzyme telomerase allows the synthesis of new telomeric DNA, thus maintaining proliferative capacity (4).

An age-related decline in telomere length may promote genetic instability and increase the risk of malignancy. Peripheral blood telomere lengths measured in individuals in their 7th decade of life is predictive of survival (5), although the strongest correlate of telomere length was mortality from cardiac and infectious disease. We have previously shown that telomere dysfunction is an early event in neoplastic progression in ulcerative colitis (6), and is related to chromosomal instability and anaphase bridge formation. This may facilitate the molecular evolution of tumorigenesis in cells by accelerating chromosomal instability (6).

We analyzed telomere length in normal colonic mucosa in individuals ages 0 to 93 years using two techniques: quantitative-fluorescence *in situ* hybridization (Q-FISH), which measures telomere length separately in epithelial and stromal cells in tissue sections, and quantitative-PCR (Q-PCR), which accurately measures telomere length using small amounts of DNA. We also examined telomere length in normal mucosa adjacent to adenomas and colorectal carcinomas in order to investigate whether a telomere-erosion field effect was evident in sporadic colorectal tumorigenesis, similar to that previously shown in ulcerative colitis (6).

Materials and Methods

Patients and Samples. Three sets of specimens were obtained. The first consisted of normal colorectal mucosa (the majority of which were rectal) from 136 subjects ranging from 0 to 93 years (55 males and 81 females) and with no history of colorectal malignancy. Diagnoses included trauma, appendicitis, diverticular disease, solitary rectal ulcer syndrome, mucosal prolapse, rectal prolapse, focal active colitis, and lymphoid tissue. Normal colonic mucosal specimens from neonates were obtained at autopsy. All analyses in this group were done on formalin-fixed tissue using Q-FISH. The second set included peripheral blood lymphocytes (PBL) and frozen normal colon biopsies from 47 patients with nonneoplastic histology, as above; 22 of these cases were from the same patients as set 1. Patients ranged from 34 to 79 years old (11 males and 36 females). Information on smoking, use of aspirin and other nonsteroidal antiinflammatory medications (NSAID), and prior gastrointestinal disease was available for 57 of 136 cases in the first set, and in 42 of 47 cases in the

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Note: J. O'Sullivan and R.A. Risques contributed equally to this work.

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second set. Aspirin or ibuprofen use was defined as ever having taken aspirin or ibuprofen at least once a week for a year or more. A nonsmoker was defined as an individual who had smoked <100 cigarettes in his or her lifetime.

The third set of specimens consisted of formalin-fixed paraffin-embedded tissue from adenomas and cancer tissue. Adenomas were categorized as either >2 cm in diameter (26 patients: 14 males and 12 females; average age, 61.2 years), 1 to 2 cm (10 patients: 8 males and 2 females; average age, 67.7 years), or <1 cm (14 patients: 11 males and 3 females; average age, 64.1 years). Cancer resections were from 12 patients (six males and six females; average age, 58 years; three with Dukes' A, four with Dukes' B, and five with Dukes' C). For 9 adenomas >2 cm and for 12 cancers, telomere length in adjacent and distant normal mucosa were also examined. These studies were done with approval from the Institutional Human Subjects Review Boards of the University of Washington, Fred Hutchinson Cancer Research Center, Group Health Cooperative, and Children's Hospital and Regional Medical Center.

Telomere Q-FISH. Dual-color (telomere and centromere) Q-FISH was done as previously described (6, 7). A minimum of 50 epithelial and stromal cells were analyzed per case to obtain an average epithelial/stromal telomere intensity (proportional to telomere length), which was calculated as the epithelial/stromal ratio (6). The intra-assay and inter-assay variability (CV) for Q-FISH was 11% and 15%, respectively.

Epithelial Cell Isolation and DNA Extraction. Epithelial and stromal cells from frozen biopsies were isolated using the epithelial shake-off method as previously described (8). Using cytokeratin staining, at least 90% of the cells were epithelial. DNA was extracted using Qiagen tissue mini-kits and from lymphocyte buffy coats using the Qiagen blood midikit (Qiagen, Valencia, CA).

Telomere Q-PCR. Telomere length was measured by Q-PCR in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) as previously described (9). A four-point standard curve (2-fold serial dilutions, 5-0.625 ng of DNA) was used to

allow transformation of cycle threshold (Ct) into nanograms of DNA. All samples were run in triplicate and the median was used for analyses. Raw data was exported to Excel and baseline background subtractions were done using a macro that aligned the amplification plots to a baseline height of 2% in the first five cycles. The fluorescence threshold for determination of the Ct was set at 20%. All standard curves showed $R^2 > 0.99$. Telomeric DNA was expressed relative to the amount of control-gene DNA (9). The intra-assay and inter-assay variability (CV) for Q-PCR was 6% and 7%, respectively.

Statistical Analysis. An unpaired *t* test was used to compare telomere length across different age groups and between adenomas and carcinomas (StatView, SAS Institute, Inc., Cary, NC). The remaining statistics were done using R (10). The association between telomere length and age was analyzed by simple linear regression and by a two-segment model consisting of two linear segments connected at a change-point in age. Resampling techniques (11) were used to determine the significance of the improvement in data fitting from the two-segment model over the one-line model, as well as the estimated confidence intervals for the slopes and the change-point in the two-segment model. Smooth curves were fit to the data points and resampling techniques were used to obtain confidence intervals. Associations between telomere length and smoking, aspirin and NSAID use, and prior gastrointestinal disease were examined in the two-segment model after adjusting for age.

Results

Telomere Length Measured by Q-FISH. Figure 1 shows representative confocal Q-FISH images of the colon of a young individual with long telomeres (A) and an older individual with shorter telomeres (B). The centromere and DNA probes served as internal controls for changes in telomere brightness that might have resulted from reduced accessibility of the telomere probe. Figure 1A illustrates similar epithelial and stromal telomere (green) and centromere

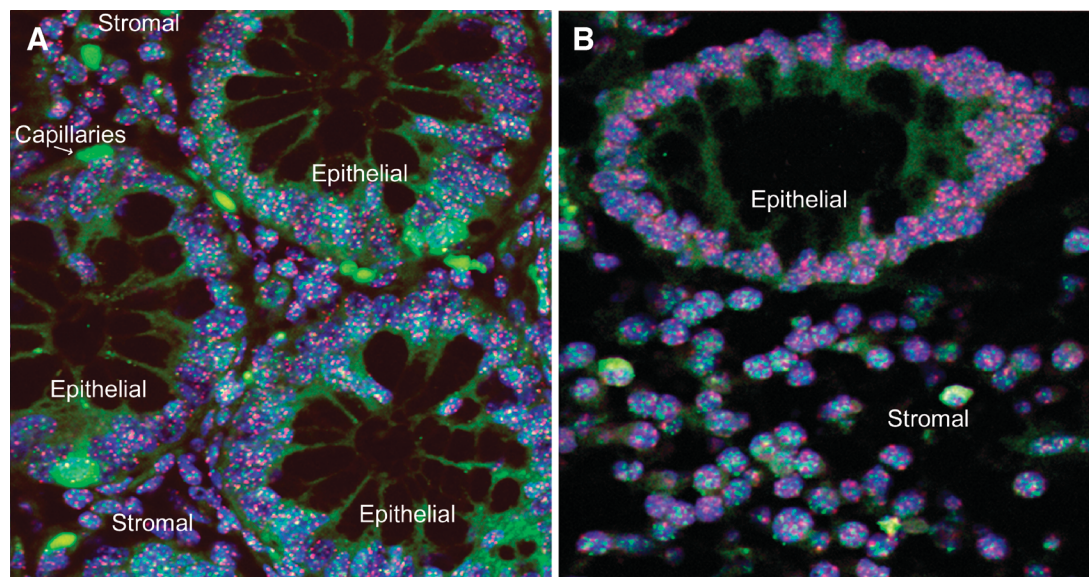


Figure 1. Q-FISH using a PNA telomere (FITC-green) and centromere probe (TAMRA-red) counterstained with a fluorescent DNA dye, TOTO-3 (false blue), in colon biopsies. **A.** Q-FISH analysis of a colon biopsy from a young donor showing equal telomere and centromere staining intensities in both the epithelial and stromal cells; no evidence of telomere shortening is seen. **B.** Telomere staining intensity in this colonic biopsy from an older donor is reduced in the epithelial cells compared with the stromal cells in this same section. No significant difference in centromere and DNA staining intensities is seen between the different cell types. Magnification, $\times 40$.

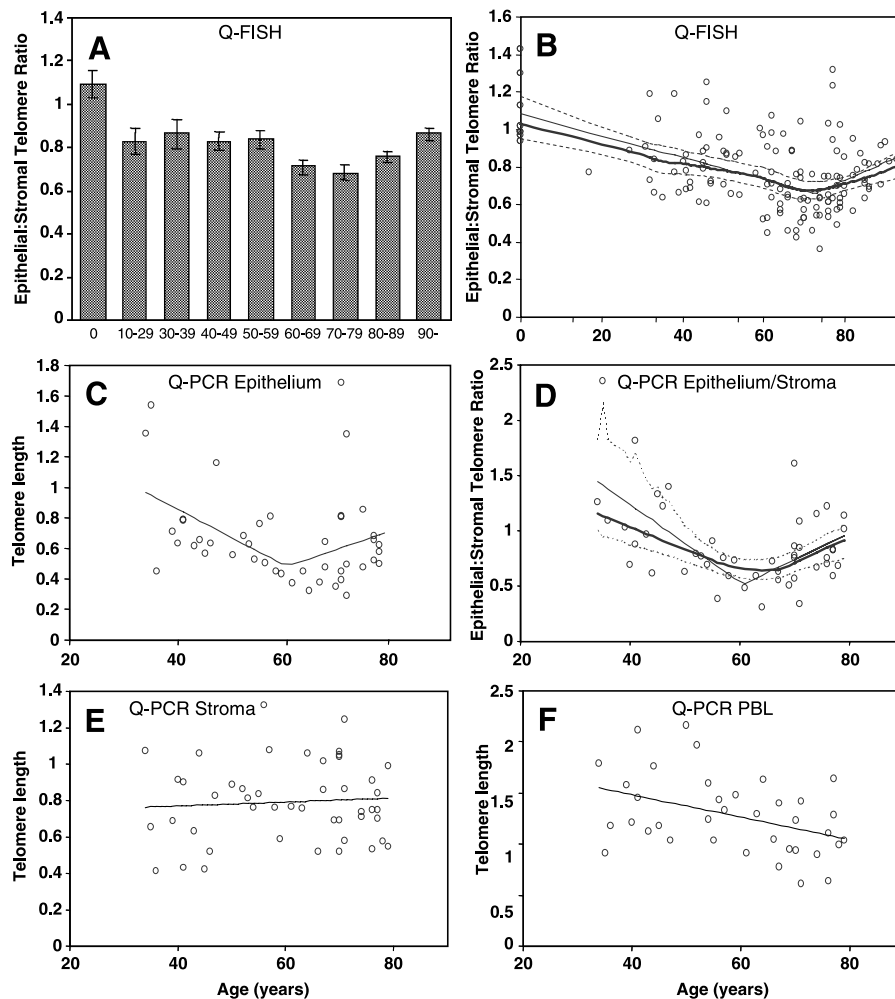


Figure 2. **A.** Telomere length measured by Q-FISH and expressed as epithelial/stromal telomere ratio (\pm SE) versus age. A general decrease in telomere length is seen with increasing age followed by an increased telomere length in the 80 to 89 and 90+ year age groups. **B.** Q-FISH telomere length versus age. Both the two-segment model (*light continuous line*) and the smoothed curve with bootstrap confidence interval (*dark continuous and dashed lines*) are indicated (see text). **C-F.** Telomere length measured by Q-PCR versus age measured in the isolated epithelium (**C**) and stroma (**E**) of normal colorectal biopsies. The epithelial/stromal telomere ratio (**D**) is related linearly to age ($P = 0.010$), but a two-segment model provides a better fit to the data ($P = 0.004$; see text). Both the two-segment model (*light continuous line*) and the smoothed curve with bootstrap confidence interval (*dark continuous and dashed lines*) are indicated. PBL telomere length (**F**) decreases linearly with age ($P = 0.011$).

(red) fluorescence intensity in cells. Figure 1B shows epithelial telomere shortening in the colonic mucosa: reduced telomere fluorescence (green) in the epithelial cells compared with the stromal cells, whereas centromere fluorescence (red) is unchanged.

Figure 2A shows the mean telomere length in 136 normal colorectal mucosa specimens. To control for differences in sample fixation, processing, and staining, data are expressed as the ratio of epithelial-to-stromal cell fluorescence (6, 7). Telomere length shortening is statistically significant for all older age groups compared with the 0- to 9-year patient group (Fig. 2A, all $P \leq 0.025$), except for the >90-year-old group ($P = 0.06$).

Although there is considerable variation in telomere length among individuals, telomere lengths in the 30 to 39, 40 to 49, and 50 to 59 year age groups, compared with those in the 60 to 69 and 70 to 79 year age groups, were all statistically different from each other (all $P < 0.05$). The only difference in telomere length in males compared with females was found in the 80 to 89 year age group, where the males showed longer telomeres ($P = 0.023$).

Figure 2B shows the relationship between telomere length and age, fitted by a line with two segments (*light continuous line*). Initially, there is an inverse association between telomere length and age; however, after 72.6 years of age, telomere length is positively associated with age. A two-segment model fits the data significantly better than a simple linear model ($P = 0.002$). The estimated 95% confidence interval for the change-point is 56.0 to 83.4. A 95% bootstrap confidence interval (*dashed lines*, Fig. 1B) for the smoothed curve (*dark continuous line*) suggests an increase in telomere length at

older ages. The telomere length variability at advanced ages (60-79 years, CV = 28%) is greater than the inter-assay variability (CV = 15%).

Telomere Length Measured by Q-PCR. The relationships between telomere length and age in colorectal epithelial and stromal cells and PBL derived using this independent method are shown in Fig. 2C-F. There is a tendency for the telomere length in the epithelium to decrease with age (Fig. 2C), but the presence of older individuals with long telomeres precludes a statistically significant correlation ($P = 0.12$, simple linear regression). A two-segment model does not fit the data better than a simple linear model ($P = 0.12$). Telomere length in stromal cells does not show any association with age, although high variability in telomere length is observed (Fig. 2E). The epithelial/stromal ratio reduces the interindividual variability and the association between this ratio and age is statistically significant ($P = 0.010$, simple linear regression; Fig. 2D). A two-segment relationship with age is also observed by Q-PCR (Fig. 2D), yielding a superior fit compared with the simple linear model ($P = 0.004$). The estimated change-point is 60.6 years (95% confidence interval, 50.0-70.4). As above, the telomere length variability at advanced ages (60-79 years, CV = 28.5%) is greater than the inter-assay variability (CV = 7%).

Telomere length in PBL measured by Q-PCR decreases linearly with age ($P = 0.011$, $R^2 = 0.17$; Fig. 2F). There is no suggestion of a change in slope at older ages; such a change in slope was also not evident when PBL telomere length was expressed as a ratio to stromal telomere length (data not shown).

Associations Between Telomere Length, Smoking, and NSAID. For Q-FISH data, smoking status and NSAID use was available for 57 out of 136 patients. No statistically significant correlations were detected for these variables. For Q-PCR data (42 out of 47 patients), there was no association between telomere length and prior gastrointestinal disease or aspirin/NSAID use. Interestingly, there was an association between telomere length and smoking: nonsmokers had epithelial/stromal telomere ratios 0.211 larger (an average of 37%) than for the same age smokers ($P = 0.03$, smoking status added to the two-segment age regression analysis).

Telomere Length in Colonic Neoplasms and Surrounding Normal Epithelium. Telomere length in variably sized adenomas (<1, 1-2, and >2 cm), colorectal cancers and normal mucosa adjacent to adenomas and cancers were assessed by Q-FISH (Table 1). In large (>2 cm) adenomas, telomeres were shorter than in normal adjacent mucosa ($P = 0.004$) and normal distant tissue ($P = 0.05$) from the same patients. There was no difference in telomere length comparing tubulovillous adenomas to villous or tubular adenomas (all $P \geq 0.17$).

In cancers, telomeres were not shorter compared with the normal adjacent, normal distant and normal age-matched control mucosa (all $P \geq 0.52$). Four of the 12 cancers had long telomeres (ratio ≥ 0.9). No association was found between telomere length and Dukes' stage (all $P \geq 0.22$).

Discussion

Q-FISH and Q-PCR showed that telomeres in human colonocytes shorten with increasing age; however, above age 60 this trend reverses, with telomere length increasing with age. Consistent with previous studies (12-16), telomere lengths show extensive heterogeneity within age groups. Telomere length has been shown to decrease with age in many tissues, at a rate from 10 to 150 bp/y (17). However, no previous reports showed an increase in telomere length in old patients, as we report here in colonocytes. This could be due to technical limitations of telomere length measurement using terminal restriction fragment analysis. Furthermore, separation of epithelial and stromal cells, not previously reported, proved to be crucial in distinguishing age-based telomere shortening in epithelial cells.

It is difficult to hypothesize a biological mechanism that would account for a progressive increase in colonocyte telomere length once individuals reach the late decades of

Table 1. Telomere length measurements in variably sized colorectal adenomas and carcinoma cases expressed as a ratio of epithelial/stromal telomere length

	Epithelial/stromal telomere ratio \pm SE	No. of patients
Adenomas		
Lesions		
<1 cm	0.750 \pm 0.06	14
1-2 cm	0.852 \pm 0.09	10
>2 cm	0.658 \pm 0.03	26
Normal adjacent		
<1 cm	0.769 \pm 0.03	36
1-2 cm	0.742 \pm 0.03	21
>2 cm	0.797 \pm 0.03	32
Normal distant		
>2 cm	0.788 \pm 0.03	9
Cancers		
Lesions	0.774 \pm 0.04	12
Normal adjacent	0.811 \pm 0.02	13
Normal distant	0.781 \pm 0.04	18

* $P = 0.004$.

† $P = 0.05$.

life. Considering the cross-sectional design of our study, an alternative interpretation is differential patient survival. Telomere length of PBL is a predictor of mortality in individuals age 60 and over (5), suggesting that individuals with shorter telomeres are less likely to survive and be studied in the late decades of life. Similarly, the risk of premature myocardial infarction (18), and bladder, head and neck, lung, and renal cancers (19) are increased in patients with shorter telomeres in peripheral blood. The subset of older individuals with shorter colorectal epithelial telomeres may be at high mortality risk. Our data would indicate that the selective survival of patients with longer colonocyte telomeres might outweigh the longitudinal effect of decreasing telomere length beginning in the seventh decade of life.

Studies have reported a significant linear decrease in telomere length in the colorectum with age (20), but these were done in noncancerous tissue from cancer patients and may not be representative of the normal population. One study has reported telomere length in normal colon from normal individuals; however, no association with age was detected, due to some old individuals with very long telomeres (21). These same individuals showed significant telomere reduction with age in the stomach, duodenum, and blood. We also observed an association between telomere length in PBL and gastric tissue (data not shown) but not between PBL and colon, suggesting that telomere behavior in colonocytes may differ from other tissues. This may be related to rates of cell proliferation relative to telomerase activity in stem cells (22) and exposure to oxidative damage (23).

We found a relationship between smoking and short telomeres in the colorectum of normal individuals by Q-PCR, suggesting that tobacco genotoxins might contribute to telomere shortening in colonocytes. This result was not observed by Q-FISH, probably due to the low number of cases in which smoking data were available.

We have shown that in large adenomas (>2 cm), there is statistically significant telomere shortening compared with normal adjacent and distant surrounding mucosa. However, no evidence of a telomere-shortening field effect was seen. These results are in agreement with previous studies showing that telomere shortening can occur at the adenoma-carcinoma transition in colorectal cancer (24). This is in contrast to our observations in ulcerative colitis, in which such a field effect was seen in patients with high-grade dysplasia or cancer (6). Genomic instability associated with critical telomere shorting may contribute to the malignant transformation of large adenomas (25). The absence of further telomere length reduction in colorectal cancers is consistent with observations that telomerase is reactivated in the large majority of colorectal cancers (22).

In conclusion, colon telomeres may be a better predictor of disease risk and death than those of PBL. Variation in telomere length between individuals may reflect genotoxic exposure history, genomic instability, and risk of disease.

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