Leukocyte Telomere Length Predicts Cancer Risk in Barrett's Esophagus

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Abstract

Purpose: **Leukocyte telomere length has gained attention as a marker of oxidative damage and age-related diseases, including cancer. We hypothesize that leukocyte telomere length might be able to predict future risk of cancer and examined this in a cohort of patients with Barrett's esophagus, who are at increased risk of esophageal adenocarcinoma and thus were enrolled in a long-term cancer surveillance program.**

Patients and Methods: **In this prospective study, telomere length was measured by quantitative PCR in baseline blood samples in a cohort of 300 patients with Barrett's esophagus followed for a mean of 5.8 years. Leukocyte telomere length hazard ratios (HR) for risk of esophageal adenocarcinoma were calculated using multivariate Cox models.**

Results: **Shorter telomeres were associated with increased esophageal adenocarcinoma risk (age-adjusted HR between top and bottom quartiles of telomere length, 3.45; 95% confidence interval, 1.35-8.78; $P = 0.009$). This association was still significant when individually or simultaneously adjusted for age, gender, nonsteroidal anti-inflammatory drug (NSAID) use, cigarette smoking, and waist-to-hip ratio (HR, 4.18; 95% confidence interval, 1.60-10.94; $P = 0.004$). The relationship between telomere length and cancer risk was particularly strong among NSAID nonusers, ever smokers, and patients with low waist-to-hip ratio.**

Conclusion: **Leukocyte telomere length predicts risk of esophageal adenocarcinoma in patients with Barrett's esophagus independently of smoking, obesity, and NSAID use. These results show the ability of leukocyte telomere length to predict the risk of future cancer and suggest that it might also have predictive value in other cancers arising in a setting of chronic inflammation.** (Cancer Epidemiol Biomarkers Prev 2007;16(12):2649–55)

Introduction

Telomeres protect the end of the chromosomes and shorten with each cell division, a process that is enhanced by oxidative stress (1). The telomere length of circulating leukocytes decreases with age, but shows a high degree of heterogeneity for a given age (2). Cross-sectional studies have shown associations between short telomeres in leukocytes and human diseases, including coronary heart disease, hypertension, and dementia, as well as associations with factors that predispose to disease, such as smoking and obesity (3). These studies support the hypothesis that mean leukocyte telomere length may be an indicator of cellular injury and repair dysfunctions that contribute to aging-related diseases. Shorter leukocyte telomeres have also been reported in patients with head and neck cancer, bladder, lung, or renal cell carcinoma compared with control subjects (4) and, recently, the association between shorter telomeres and risk of bladder cancer was confirmed in two larger nested case-control studies (5). As Barrett's esophagus is one of the best established examples of chronic inflammatory disease that predisposes to cancer, it is an excellent model in which to analyze the hypothesis that leukocyte telomere length is a biomarker of cancer risk in the setting of chronic inflammation.

Barrett's esophagus is a chronic active inflammatory condition in which the normal squamous epithelium is replaced by a metaplastic columnar epithelium, usually as a consequence of chronic gastroesophageal reflux disease (6). Barrett's esophagus is the only known precursor of esophageal adenocarcinoma, a cancer that is on an exponential increase (7) and has very poor prognosis unless detected early (all-stage 5-year survival is 15%; ref. 8). Approximately 0.5% to 1% of Barrett's esophagus patients per year will develop esophageal adenocarcinoma and, currently, the only way to identify those patients is through periodic endoscopic biopsy surveillance, which is expensive, time-consuming, and of questionable effectiveness (9, 10). If improved risk biomarkers were found, surveillance and prevention efforts could be focused on the subset of patients with highest risk, substantially reducing cost, patient anxiety, and potential morbidity.

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The risk of esophageal adenocarcinoma is affected by environmental factors that, interestingly, are also known to be related to leukocyte telomere length. Smoking and obesity are the strongest risk factors for esophageal adenocarcinoma (11, 12) and have also been reported to be associated with telomere attrition with age (13). Nonsteroidal anti-inflammatory drug (NSAID) use protects against development of esophageal adenocarcinoma in patients with Barrett's esophagus (14, 15), even in patients with high-grade dysplasia and high-risk molecular abnormalities (16). The protective effect of NSAID use is likely to be related to reduced levels of inflammation and oxidative damage as well as reduced cellular proliferation, events that shorten telomere length (1) and are believed to have a causative role in the progression of Barrett's esophagus to esophageal adenocarcinoma (17). Therefore, obesity, smoking, and NSAID use are important environmental factors that might interact with leukocyte telomere length to predict the predisposition of a patient to develop esophageal adenocarcinoma.

We hypothesized that, as a potential integrative measure of a patient's history of inflammation and oxidative damage resulting from both environmental and intrinsic factors, telomere length in leukocytes of Barrett's esophagus patients might predict the risk of progression to esophageal adenocarcinoma. To test this, we measured leukocyte telomere length in a cohort of 300 individuals with Barrett's esophagus followed prospectively over an average of 5.8 years.

Patients and Methods

Study Participants. Patients were enrolled in the Seattle Barrett's Esophagus Research Program, a dynamic cohort study that began in 1983 and has been approved annually by Human Subjects Review Boards at the University of Washington and/or the Fred Hutchinson Cancer Research Center. Three hundred participants were eligible, as defined by the diagnosis of specialized intestinal metaplasia in esophageal biopsies, with no history of esophageal malignancy, with at least one follow-up endoscopy, and with a baseline blood sample available. Baseline was defined as the first endoscopy between January 5, 1995, and December 2, 1999. Patients underwent serial endoscopies for a total of 1,741 patient years of follow-up (mean 5.8 years, range 0.1-11.1 years). Follow-up time was calculated between baseline and the last endoscopy before June 2006 or the first endoscopy with esophageal adenocarcinoma. This study was conducted at a specialty research and referral center, and thus our cohort is considered a high-risk patient population. We have included all esophageal adenocarcinomas that developed subsequent to the baseline evaluation so that accurate risk stratification models can be developed based on findings at a single baseline endoscopy (18). We conducted intensive endoscopic surveillance within 4 months of the baseline of any patient with high-grade dysplasia (19). Exclusion of patients who were diagnosed with esophageal adenocarcinoma that developed before 4 months from baseline ($n = 6$) did not alter the conclusions of the study. History of smoking was evaluated as ever versus never smoker and as current, former, and never smoker. Ever smoker was defined as an individual who smoked at least one cigarette a day for at least 6 months. The assessment of NSAID use was as previously described (14, 20). NSAID user was defined as an individual who used NSAID at least once a week for at least 6 months at the time of baseline, within 1 year before baseline or any time during follow-up (excluding follow-up after attainment of end point). Anthropometric measurements, including body mass index (BMI) and waist-to-hip ratio (WHR), were taken at baseline and at follow-up by use of a standard protocol (15). Written informed consent was obtained from all participants.

Telomere Quantitative PCR. Buffy coats were prepared from baseline blood samples after hypotonic red cell lysis and stored at −80°C. DNA was extracted using the salting out method (21). Telomere length was measured by quantitative PCR (22). Each sample was amplified for telomeric DNA and for 36B4, a single-copy control gene that provided an internal control to normalize the starting amount of DNA. A four-point standard curve (1.6-fold serial dilutions from 10 to 2.44 ng DNA) was used to transform cycle threshold into nanograms of DNA. Baseline background subtraction was done by aligning amplification plots to a baseline height of 2% in the first five cycles. The cycle threshold was set at 20% of maximum product. All samples were run in triplicate and the median was used for calculations. The amount of telomeric DNA (T) was divided by the amount of single-copy control gene DNA (S), producing a relative measurement of the telomere length (T/S ratio). Two control samples were run in each experiment to allow for normalization between experiments, and periodic reproducibility experiments were done to guarantee correct measurements. The intra-assay and inter-assay variability (coefficient of variation) for quantitative PCR was 6% and 7%, respectively. Because the T/S ratio is a relative measure of telomere length, the mean of T/S ratio of the cohort was normalized to 1.0 to facilitate comparisons.

Statistical Analysis. Linear regression was used to evaluate the relationship between telomere length and age. As the distribution of telomere length was confirmed to be normal, comparison of means between groups was done with nonpairwise two-sided t tests. For these comparisons, telomere length values were age-adjusted using the slope parameter of the age versus telomere length regression, and the P values were adjusted for multiple comparisons (23). Leukocyte telomere length hazard ratios (HR) and 95% confidence intervals (95% CI) for risk of esophageal adenocarcinoma were estimated using multivariate Cox regression models. The Kaplan-Meier survival curve method was used to depict the cumulative cancer incidence over time. All analyses were carried out with Statistical Analysis System software (version 9.1, SAS Institute, Inc.).

Results

Telomere Length Associations with Host and Environmental Factors at Baseline. Table 1 shows the cohort characteristics at baseline and the association of leukocyte telomere length with each of these factors. The linear regression of leukocyte telomere length with age was strong and statistically significant [$r = -0.28$,

Table 1. Associations between leukocyte telomere length and host and environmental factors at baseline

Variable	No. patients	Mean age	Relative telomere length mean (SE)	*P*
Age (y)				<0.0001
30-49	44	43.1	1.139 (0.029)	
50-69	169	59.3	1.026 (0.015)	
70-89	87	76.1	0.880 (0.020)	
Gender				0.027
Male	241	61.4	0.990 (0.011)*	
Female	59	62.0	1.039 (0.022)*	
NSAID use				0.72
User	105	64.2	0.985 (0.018)*	
Nonuser	181	59.6	1.009 (0.013)*	
Tobacco use				0.53
Ever	188	61.7	0.994 (0.011)*	
Never	99	60.5	1.011 (0.021)*	
WHR				0.19
≤0.910	73	58.7	1.047 (0.019)*	
0.910-0.952	71	62.2	0.965 (0.016)*	
0.952-0.991	74	61.4	0.990 (0.015)*	
>0.991	69	62.6	0.996 (0.016)*	
BMI (kg/m^2)				0.12
≤25	42	65.7	0.973 (0.028)*	
26-30	135	61.7	0.988 (0.010)*	
>30	105	59.2	1.027 (0.014)*	

NOTE: All *P* values are adjusted for multiple comparisons.
Abbreviation: BMI, body mass index.
*Age adjusted.

slope = −0.0036 (95% CI, −0.0050 to −0.0021), $P < 0.0001$], corresponding to a decrease of 0.36% per year. Therefore, all other telomere length analyses presented in the table were age adjusted. Significantly shorter telomeres were observed in males than in females ($P = 0.027$), but associations between telomere length and NSAID use, smoking, and obesity, as measured by WHR and body mass index, were not statistically significant (Table 1).

Cancer Risk Prediction by Telomere Length. Thirty-eight participants developed esophageal adenocarcinoma during the course of surveillance. Leukocyte telomere length at baseline was a strong predictor of cancer risk as shown by univariate and multivariate Cox model analysis (Table 2). When patients were divided into four groups based on their telomere length, we observed a consistent increase in the risk of cancer corresponding to the decrease of leukocyte telomere length over the four groups. The group of patients with the shortest telomeres had the highest risk of developing cancer compared with the group of patients with the longest telomeres (HR, 4.59; 95% CI, 1.40-17.32; $P = 0.02$). This association was still significant after adjustment simultaneously or individually for age and for all the other risk factors considered in this study, including gender, NSAID use, smoking, and WHR (Table 2). When telomere length was treated as a continuous variable, the HR between the fourth and first quartiles of telomere length was 3.93 (95% CI, 1.59-9.70; $P = 0.003$) and similar significant HR were observed after adjustment for age (HR, 3.45; 95% CI, 1.35-8.78; $P = 0.009$) and for age, gender, NSAID use, cigarette smoking, and WHR (HR, 4.18; 95% CI, 1.60-10.94; $P = 0.004$). Further adjustment for body mass index and smoking categorized into three groups (current, former, never) did not significantly change the HR (data not shown). The cancer cumulative incidence curves for each quartiles of telomere length are shown in Fig. 1.

Modification of Telomere Length Cancer Risk by Host and Environmental Factors. Because NSAID use protects from esophageal adenocarcinoma and smoking and obesity predispose to esophageal adenocarcinoma, we evaluated whether the prognostic value of leukocyte telomere length varied in these subgroups of patients. Therefore, we stratified the cohort according

Table 2. Univariate and multivariate Cox regression analysis

Telomere length	No. patients (EA/total)	Unadjusted		Age adjusted		Adjusted for other risk factors*	
		HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Categorical variable							
First quartile (longest)	4/75	1.00		1.00		1.00	
Second quartile	9/75	2.57 (0.72-10.78)	0.17	2.40 (0.62-9.32)	0.21	2.39 (0.61-9.34)	0.21
Third quartile	10/75	3.27 (0.93-12.34)	0.07	2.99 (0.82-10.98)	0.10	3.49 (0.94-12.87)	0.06
Fourth quartile (shortest)	15/75	4.59 (1.40-17.32)	0.02	3.99 (1.11-14.33)	0.03	4.66 (1.28-16.93)	0.02
Continuous variable							
Fourth vs first quartile †	38/300	3.93 (1.59-9.70)	0.003	3.45 (1.35-8.78)	0.009	4.18 (1.60-10.94)	0.004

Abbreviation: EA, esophageal adenocarcinoma.
*Age, gender, NSAID use, smoking, and WHR.
†Median of fourth quartile versus median of first quartile.

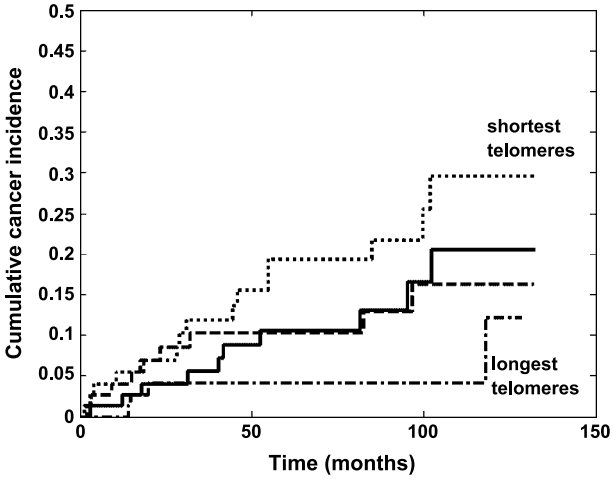

Figure 1. Cancer incidence in Barrett's esophagus patients stratified by quartiles of leukocyte telomere length measured at baseline. *Dashed-dotted line,* first quartile (longest telomeres); *dashed line,* second quartile; *black line,* third quartile; *dotted line,* fourth quartile (shortest telomeres).

to categories of NSAID use, smoking, and WHR, and used Cox models to determine the HR associated with short telomeres (Table 3). Kaplan-Meier curves were used to depict the cumulative cancer incidence for each category (Fig. 2). We used tertiles rather than quartiles to allow sufficient numbers of patients in each of the categories. First, we compared the risk effects of telomere length within groups of each of the host and environmental factors. Shorter leukocyte telomeres were strongly associated with cancer risk in NSAID nonusers, smokers, and individuals with low WHR, but not in any of the other subgroups (Table 3; Fig. 2). For NSAID nonusers, the HR between the third and first tertiles of telomere length was 2.94 (95% CI, 1.33-6.52; $P = 0.008$), for smokers was 4.74 (95% CI, 1.77-12.69; $P = 0.002$), and for individuals with low WHR was 4.25 (95% CI, 1.28-14.16; $P = 0.02$). We also evaluated the risk effects of telomere length between groups of each category. Specifically, we observed that NSAID use had the greatest protective effect among the patients with the shortest telomeres (Fig. 2A versus B; log-rank test, $P = 0.04$). Similarly, the greatest increased cancer risk from smoking seems to be among the patients with the shortest telomeres (Fig. 2C versus D; log-rank test, $P = 0.05$). Patients with long leukocyte telomeres and low WHR seem to be at little risk of cancer within 10 years (Fig. 2E).

Because the previous results suggested that a patient's outcome was related to telomere length as well as to environmental factors, we explored combined models that could comprehensively assess the HR of a patient according to his or her anthropometric and environmental factors and leukocyte telomere length. The cancer HR of a person with high WHR, a NSAID nonuser, and a smoker versus a person with low WHR, a NSAID user, and a nonsmoker was 7.9 (95% CI, 3.0-47.1). If we added telomere length to this model, then we could discriminate a subset of patients with even higher risk: A patient with high WHR, a non-NSAID user, a smoker, and with short leukocyte telomeres has a 20.6-fold (95% CI, 3.8-111.9) higher risk of developing esophageal adenocarcinoma than a patient with low WHR, a NSAID user, a nonsmoker, and with long leukocyte telomeres.

Discussion

Our results show that telomere length measured in the blood of patients with Barrett's esophagus predicts their risk of subsequent esophageal adenocarcinoma. Telomere length was a significant risk factor after adjusting for all other predictors of esophageal adenocarcinoma, including gender, age, smoking, WHR, and NSAID use. Moreover, we observed that the patients that have the shortest telomeres in leukocytes are at greatest risk due to smoking and are also those that benefit most from the protective effect of NSAID use. This suggests that short telomeres are a strong marker of cancer susceptibility, but that this risk can be modified by factors that promote (smoking) or prevent (NSAID) tumor evolution. Accordingly, a combined estimate of leukocyte telomere length and host and environmental factors is a more optimal predictor of the risk of cancer in patients with Barrett's esophagus, as shown by the higher HR of the combined models.

One of the strengths of this study is its longitudinal design. The cohort included 300 patients that have been followed for an average of 5.8 years. It is a high-risk cohort, as evidenced by the number of patients who progressed to cancer during the course of this study, which allowed stratification of risk according to categorical variables. With 38 esophageal adenocarcinoma cases, this study is second only to our previous 15-year report of histology and flow cytometry (42 esophageal adenocarcinoma cases; ref. 18), and substantially larger than any other longitudinal study of biomarkers in Barrett's esophagus (24-28). Another strength of this study is that its design addressed the most common pitfalls in telomere epidemiology studies (29): We used a sufficient sample size; did a strict quality control of telomere length measurements; and controlled for potential confounders, including age, gender, smoking, obesity, and NSAID use. Studies in other centers, however, will be required to confirm that our results can be generalized to other patient populations. If so, leukocyte telomere length combined with other host and environmental risk factors

Table 3. Cancer HRs by telomere length stratified by host and environmental factors

Variable	No. patients (EA/total)	Estimation by Cox model*	
		HR† (95% CI)	*P*
NSAID use			
User	7/105	4.26 (0.43-42.0)	0.21
Nonuser	26/181	2.94 (1.33-6.52)	0.008
Tobacco use			
Never	6/99	1.58 (0.41-61.74)	0.51
Ever	27/191	4.74 (1.77-12.69)	0.002
WHR			
High (>0.952)‡	20/143	2.57 (0.98-6.72)	0.054
Low (<0.952)	13/144	4.25 (1.28-14.16)	0.020

*For telomere length as a continuous variable and adjusted for age.
†Median of third tertile versus median of first tertile.
‡Cutoff point for WHR ratio corresponds to the median value.

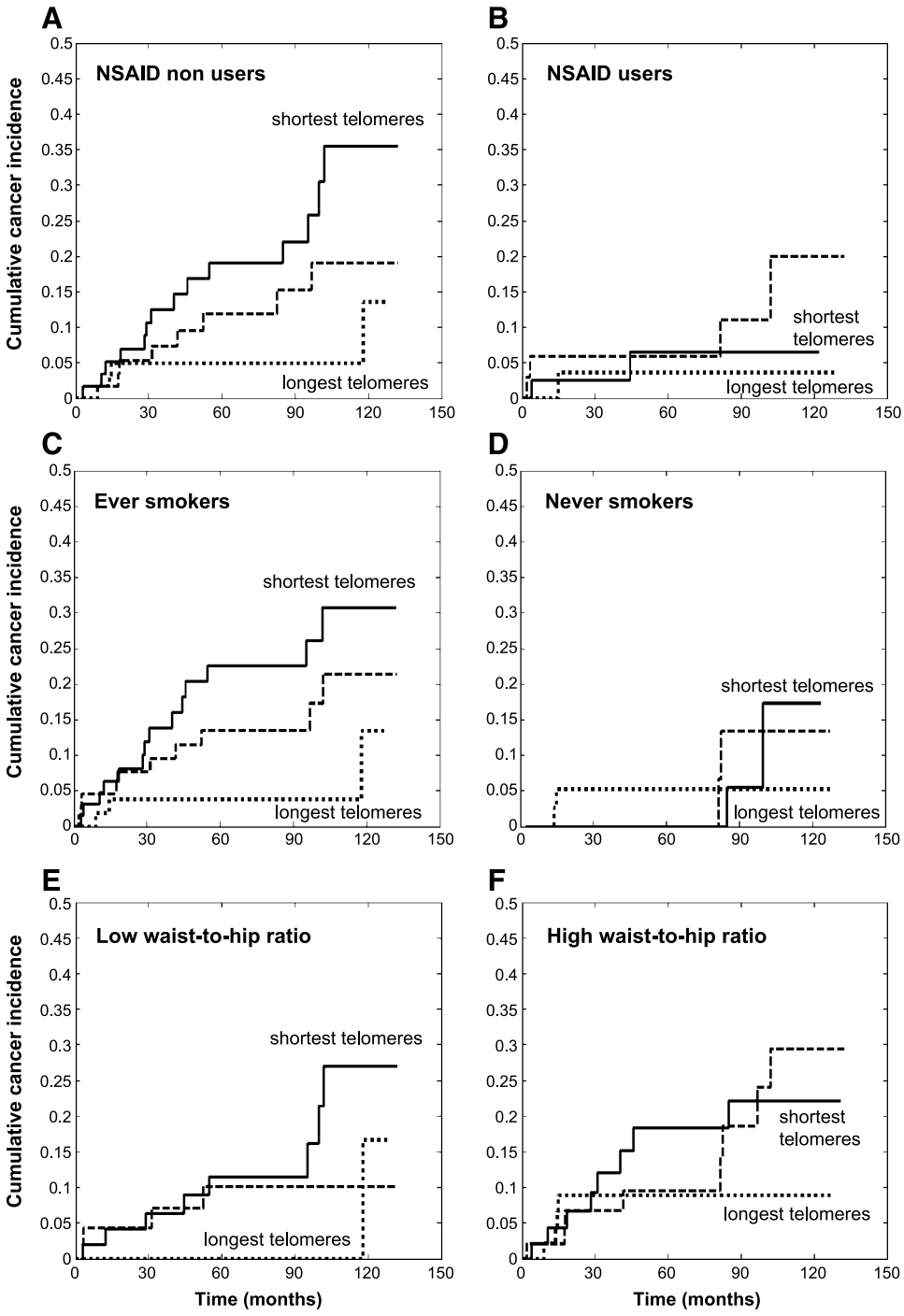

Figure 2. Cancer incidence curves for tertiles of leukocyte telomere length within subsets of patients stratified by host and environmental variables. *Dotted line,* first tertile (longest telomeres); *dashed line,* second tertile; *black line,* third tertile (shortest telomeres). **A,** NSAID nonusers. **B,** NSAID users. **C,** ever smokers. **D,** never smokers. **E,** low WHR (<0.952). **F,** high WHR (>0.952). The cutoff point for WHR ratio corresponds to the median value.

could help provide more accurate and less invasive methods to predict cancer risk in patients with Barrett's esophagus.

Telomere shortening has been reported to be one of the earliest and most prevalent alterations in epithelial carcinogenesis (30), contributing to the acquisition of chromosomal instability (31) and, in turn, promoting tumor evolution (32). Telomere shortening has been observed in gastroesophageal reflux disease (33), which precedes Barrett's esophagus, and also in the early stages of Barrett's esophagus, in association with chromosomal instability (34). However, in dysplastic Barrett's esophagus biopsies, telomere length tended to increase (34), in agreement with previous studies that reported increased expression of both RNA template (35) and the reverse transcriptase catalytic subunit (36) components of telomerase in Barrett's esophagus dysplasia. Telomerase activity, however, is not always associated with the presence of dysplasia in Barrett's esophagus (37). Given the complex dynamics of telomere length and telomerase

activity in Barrett's esophagus epithelium, leukocyte telomeres might be a more consistent prognostic marker, which could also reduce the frequency of endoscopic surveillance.

The mechanism that underlies the association between shorter telomeres in leukocytes and cancer is unknown, but at least two explanations are possible. The first one is that both are consequence of oxidative damage. Several lines of evidence indicate that oxidative stress plays a causal role in the development of Barrett's esophagus and in its progression to esophageal adenocarcinoma: (*a*) oxidative damage was increased in the esophagus of rats with induced esophageal adenocarcinoma (38) and rats with induced reflux esophagitis (39, 40); (*b*) Barrett's esophagus mucosa shows increased levels of reactive oxygen species (17) and DNA adducts (41), and decreased antioxidant capacity (42); and (*c*) higher intakes of antioxidants (43) and use of NSAID (14) are associated with decreased risk of esophageal adenocarcinoma. Oxidative stress is also one major cause of telomere shortening because single-strand breaks formed by oxidative or alkylative DNA damage are not repaired well in telomeres (1). Therefore, in patients with Barrett's esophagus, oxidative stress due to inflammation or exposure to environmental oxidants might promote the development of cancer while it accelerates the age-associated shortening of leukocyte telomeres. However, it is also possible that short telomeres could be a constitutional feature of the individuals at risk for esophageal adenocarcinoma. Telomere length has a strong genetic component (44) and, for a given age, telomere length in healthy donors shows high heterogeneity (2). Shorter telomeres have been found not only in leukocytes of individuals with neck, bladder, lung, and kidney cancers compared with normal controls (4, 5), but also in buccal cells of bladder cancer patients compared with control subjects (45), consistent with a predisposition to cancer due to constitutionally short telomeres. Furthermore, in the Li-Fraumeni syndrome, shorter telomeres are associated with the presence of cancer and with a younger age of cancer initiation (46). The above explanations are not mutually exclusive, as shorter telomeres in leukocytes of Barrett's esophagus patients that develop cancer might reflect both constitutionally short telomeres and further shortening by oxidative damage. The fact that NSAID use seems to reduce the elevated risk of esophageal adenocarcinoma associated with telomere shortening indicates, however, that genetically short telomeres are not the only factor that drives this relationship. Thus, we favor the explanation that leukocyte telomere shortening in Barrett's esophagus patients is a consequence of inflammation and oxidative damage, supporting its role as a potential marker of cumulative exposure to stress and risk of aging diseases, as previously suggested (3, 47). Further studies using other constitutional tissues, cell subsets of leukocytes, and longitudinal blood samples are under way and promise to add light to these issues.

In summary, we have shown that Barrett's esophagus patients who have short telomeres are at higher risk of developing esophageal adenocarcinoma, demonstrating the ability of leukocyte telomere length to predict cancer risk in a setting of chronic inflammation. This finding has obvious potential as part of a cancer risk model to identify individuals with Barrett's esophagus who are at increased risk of developing esophageal adenocarcinoma and to reduce the frequency of surveillance in low-risk populations. In addition, our results support the prevalent idea that leukocyte telomere length acts as an indicator of the replicative history and cumulative level of oxidative stress in the individual (47). Many human cancers are preceded by an inflammatory condition (48, 49) that is usually associated with increased levels of oxidative damage (50). Although adenocarcinoma in Barrett's esophagus is a relatively uncommon disease, the possibility that leukocyte telomere length might have prognostic value for other types of cancers related to chronic inflammation deserves further investigation.

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