Telomere Dysfunction: A Potential Cancer Predisposition Factor

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Background: **Genetic instability associated with telomere dysfunction (i.e., short telomeres) is an early event in tumorigenesis. We investigated the association between telomere length and cancer risk in four ongoing case–control studies.** ***Methods:*** **All studies had equal numbers of case patients and matched control subjects (92 for head and neck cancer, 135 for bladder cancer, 54 for lung cancer, and 32 for renal cell carcinoma). Telomere length was measured in peripheral blood lymphocytes from study participants. Genetic instability was assessed with the comet assay. Patient and disease characteristics were collected and analyzed for associations with risk for these cancers. All statistical tests were two-sided.** ***Results:*** **Telomeres were statistically significantly shorter in patients with head and neck cancer (6.5 kilobases [kb]) than in control subjects (7.4 kb) (difference = 0.9 kb, 95% confidence interval [CI] = 0.5 to 1.2 kb; $P<.001$). Nine percent of patients with head and neck cancer were in the longest quartile of telomere length, whereas 59% were in the shortest quartile. Similar patterns were observed for lung, renal cell, and bladder cancer. When subjects were categorized into telomere length quartiles defined by the distribution in control subjects, the following inverse relationship between telomere length and cancer risk was observed: adjusted odds ratios [ORs] for decreasing quartiles = 0.84 (95% CI = 0.36 to 1.97), 1.77 (95% CI = 0.72 to 4.36), and 5.11 (95% CI = 1.90 to 13.77). In stratified analysis, we found a suggestive greater-than-additive interaction between smoking status and telomere length: for ever smokers with short telomeres, OR = 25.05 (95% CI = 6.91 to 90.73); for never smokers with short telomeres, OR = 6.18 (95% CI = 1.72 to 22.13); and for ever smokers with long telomeres, OR = 6.49 (95% CI = 1.54 to 27.38). Telomere length was statistically significantly and inversely associated with baseline and mutagen-induced genetic instability.** ***Conclusion:*** **Short telomeres appear to be associated with increased risks for human bladder, head and neck, lung, and renal cell cancers.** **[J Natl Cancer Inst 2003;95:1211–18]**

Genetic integrity is maintained, in part, by the architecture of telomeres. Telomeres are TTAGGG repeat complexes bound by specialized nucleoproteins at the ends of chromosomes in all eukaryotic cells *(1,2).* By capping the ends of chromosomes, telomeres prevent nucleolytic degradation, end-to-end fusion,

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irregular recombination, and other events that are normally lethal to a cell *(3).* This genetic integrity, however, is gradually lost as telomeres progressively shorten with each cell replication cycle. This telomere shortening is a result of end-replication problems caused by DNA polymerase having difficulty replicating the very ends of linear DNA *(4,5).* Telomere shortening may induce cells to undergo apoptosis or may induce chromosomal instability *(6).* In fact, telomere dysfunction (short telomeres) has been associated with the initiation and progression of mouse and human intestinal neoplasia *(7).* Dysfunctional telomeres may also increase the risk of developing epithelial cancers by a process of breakage-fusion-bridge that leads to the formation of complex nonreciprocal translocations (a classical cytogenetic feature of human carcinoma) *(8).*

Although telomere shortening is inversely associated with age, telomere length has been found to vary considerably in human peripheral blood lymphocytes from individuals of the same age *(9).* Our hypothesis was that individuals with telomere dysfunction may be at higher risk for developing cancer and more likely to exhibit genetic instability. To test this hypothesis, we investigated whether telomere dysfunction, as assessed by telomere length, was associated with the risk of four smoking-related cancers—head and neck, bladder, lung, and renal cell cancer—in four ongoing case–control studies.

Materials and Methods

Study Population

Four case–control studies of head and neck, bladder, lung, and renal cell cancer were included in this study (Table 1). All case patients with bladder, lung, or renal cell carcinoma were recruited from The University of Texas M. D. Anderson Cancer Center through a daily review of computerized appointment schedules. They had been diagnosed within 1 year of recruitment, had histologic confirmation of their cancer, and had had no prior chemotherapy or radiotherapy. Case patients with head and neck cancer were recruited for a chemoprevention program from The University of Texas M. D. Anderson Cancer Center's Community Clinical Oncology Program affiliates and from Radiation Therapy Oncology Group centers throughout the country. There were no age, sex, or stage restrictions. Control subjects with no prior history of cancer were identified from the rosters of Kelsey Seybold, the largest multispecialty physician group in Houston, TX. Control subjects were matched to the case patients by age (±1 year), sex, and ethnicity. Some control subjects for bladder, lung, and renal cell carcinoma were shared across the studies, because they were drawn from the same control subject pool and because the same quantitative fluorescence *in situ* hybridization laser scanning cytometry (Q-FISH^{LSC}) technique was used to measure telomere length. For instance, 30% of the control subjects were shared between the lung and bladder cancer studies, 11% of the control subjects were shared between the bladder and renal cell carcinoma studies, and 19% of the control subjects were shared between the lung cancer and renal cell carcinoma studies. However, control subjects for the head and neck cancer study could not be shared across studies because a different assay was used to measure telomere length. Signed informed consent was obtained from each individual. All participants were interviewed to collect information regarding demographics, smoking history, alcohol consumption, family cancer history, medical history, and working history (except for the head and neck cancer study). Baseline blood samples were collected after the interview. This research was approved by all relevant review boards and was in accord with an assurance filed with, and approved by, the U.S. Department of Health and Human Services.

Table 1. Distribution of select characteristics of case patients and control subjects*

Variables	Case patients	Control subjects	*P* value†
Head and neck cancer			
Sex, No. (%)			
Men	88 (96)	88 (96)	
Women	4 (4)	4 (4)	1.000
Age, y (SD)	57.6 (9.7)	57.4 (9.74)	.904
Smoking status, No. (%)			
Never	14 (15)	38 (41)	
Former	42 (46)	44 (48)	
Current	36 (39)	10 (11)	<.001
No. of pack-years (SD)	42.6 (36.8)	14.0 (23.4)	<.001
Bladder cancer			
Sex, No. (%)			
Men	102 (76)	102 (76)	
Women	33 (24)	33 (24)	1.000
Age, y (SD)	63.9 (10.8)	63.7 (10.7)	.905
Smoking status, No. (%)			
Never	36 (27)	65 (48)	
Former	63 (47)	57 (42)	
Current	36 (27)	13 (10)	<.001
No. of pack-years (SD)	28.9 (30.2)	13.3 (22.5)	<.001
Lung cancer			
Sex, No. (%)			
Men	37 (69)	37 (69)	
Women	17 (31)	17 (31)	1.000
Age, y (SD)	64.7 (8.9)	64.5 (9.0)	.914
Smoking status, No. (%)			
Never	3 (5)	26 (48)	
Former	28 (52)	23 (43)	
Current	23 (43)	5 (9)	<.001
No. of pack-years (SD)	53.9 (38.3)	16.3 (23.5)	<.001
Renal cell carcinoma			
Sex, No. (%)			
Men	24 (75)	24 (75)	
Women	8 (25)	8 (25)	1.000
Age, y (SD)	59.2 (8.6)	59.1 (8.6)	.988
Smoking status, No. (%)			
Never	5 (16)	16 (50)	
Former	19 (59)	10 (31)	
Current	8 (25)	6 (19)	.012
No. of pack-years (SD)	25.1 (20.8)	17.4 (26.4)	.197

*SD = standard deviation.

†All statistical tests were two-sided.

The head and neck cancer study included 92 case patients and 92 matched control subjects. The bladder cancer study included 135 case patients and 135 control subjects. The lung cancer study included 54 case patients and 54 control subjects. The renal cell carcinoma study included 32 case patients and 32 control subjects. Case patients and control subjects were well matched with respect to age (±1 year), sex, and ethnicity. All subjects were Caucasian. As predicted, there were more current smokers and a higher number of pack-years smoked among case patients than among control subjects.

Blood Collection and Lymphocyte Isolation

Forty milliliters of blood was collected in tubes containing sodium heparin. The blood samples were coded before delivery (or mailing for the head and neck cancer study) to the laboratory

so that researchers performing the assays were blinded to the status of the samples (i.e., case or control). Lymphocytes were isolated by use of Ficoll-Hypaque centrifugation. An aliquot of 4×10^6 isolated lymphocytes was placed into a vial, frozen in 50% fetal bovine serum, 40% RPMI-1640 medium, and 10% dimethyl sulfoxide (Fisher Scientific, Pittsburgh, PA) and stored in liquid nitrogen.

Telomere Length Measurement by Q-FISH^{LSC}

The frozen lymphocytes were thawed and incubated in RPMI-1640 medium supplemented with 20% fetal bovine serum and phytohemagglutinin (60 μg/mL; Murex Diagnostics, Norcross, GA) at 37 °C for 72 hours. Slides of cell suspensions were prepared and aged for 2–4 hours. Telomere length was measured by an improved Q-FISH^{LSC} method, which was modified from a Q-FISH flow cytometry method *(10)* that used a fluorescence-labeled peptide-coupled nucleic acid (PNA) probe (Applied Biosystems, Foster City, CA). Briefly, a mixture of 3 μL of PNA probe and 7 μL of LSI hybridization buffer (Vysis, Downers Grove, IL) was applied to the target area of the slide. Slides were heated on a heating block at 74 ± 1 °C for 4.5 minutes to denature genomic DNA and were placed in a humidified hybridization chamber at room temperature for 2 hours. After incubation, slides were washed in a prewarmed solution of 1× phosphate-buffered saline (PBS; pH 7.4) and 0.1% Tween 20 at 57 ± 1 °C for 30 minutes followed by a 1-minute rinse in a solution of 2× standard saline citrate and 0.1% Tween 20. Propidium iodide was added to the slides for counterstaining the nuclei. The slides were ready for quantification after 15 minutes. The fluorescence signal was measured by laser scanning cytometry (LSC; CompuCyte, Cambridge, MA). At least 2500 cells were measured per sample. The telomere fluorescence signal was defined as the mean fluorescence signal in cells from each sample. The relative telomere length was calculated as the ratio between the telomere signal of each sample and that of the control cell line (LW5770, a lymphoblastoid cell line established in our laboratory).

Telomere Length Measurement by Southern Blot Analysis

One microgram of purified genomic DNA was first digested with *Hin*fI and *Rsa*I, and fragments were separated by agarose gel electrophoresis in 0.8% gels. DNA fragments were then transferred to a nylon membrane and hybridized to a digoxigenin-labeled probe specific to telomeric repeats, followed by incubation with a digoxigenin-specific antibody covalently coupled to alkaline phosphatase. Finally, the immobilized telomere probe was visualized with alkaline phosphatase-metabolizing CDP-Star (Roche Molecular Biochemicals, Mannheim, Germany), a highly sensitive chemiluminescent substrate. The average length of telomere restriction fragments was determined by comparing the signal with a molecular weight standard.

Comet Assay

Blood lymphocyte cultures were established as soon as the blood samples were received. Three separate cultures for each study subject were set up to measure comets from untreated baseline and mutagen-treated cells. After a 72-hour incubation, one culture was processed for the comet assay without any mutagen treatment, and another culture was irradiated at 150 rad with a ^{137}Cs source at room temperature immediately before the comet assay. After a 48-hour incubation, the third culture received 10 μL of 0.4 m*M* benzo[*a*]pyrene diol epoxide (BPDE), and the incubation was continued for another 24 hours before the comet assay. Fully frosted slides were precoated on each end of the slide with 50 μL of 1% agarose in PBS and covered with a glass coverslip (22 × 22 mm); 50 μL of blood culture was gently mixed with 150 μL of 0.5% low-melting-point agarose (Invitrogen, Carlsbad, CA) spread onto each end of the precoated slide and then covered with a fresh glass coverslip. A final layer of 0.5% low-melting-point agarose in PBS was applied to the slide and covered with a new glass coverslip. The cells were lysed by submersion in freshly prepared 1× lysis buffer (2.5 *M* NaCl, 100 m*M* EDTA, 1% *N*-lauroylsarcosine sodium salt, and 10 m*M* Tris, adjusted to pH 10 with NaOH; 10% dimethyl sulfoxide and 1% Triton X-100 were added before use) for 1 hour at 4° C. To allow DNA denaturation, unwinding, and exposure of the alkali-labile sites, the slides were placed in a horizontal electrophoresis box without power that was filled with freshly prepared alkali buffer (300 m*M* NaOH and 1 m*M* EDTA at pH>13) for 30 minutes at 4 °C. To separate the damaged DNA from the intact nuclei, a constant electric current of 295–300 mA was applied for 23 minutes at 4° C. After electrophoresis, the slides were neutralized in 0.4 *M* Tris–HCl (pH 7.4) for 5 minutes at room temperature and fixed in 100% methanol for 5–10 minutes. DNA damage in the individual cells was then visualized under a fluorescence microscope and automatically quantified via Komet 4.0.2 (Kinetic Imaging Ltd., Wirral, U.K.) imaging software attached to the microscope. Fifty consecutive cells (25 cells from each end of the slide) were imaged, and comet cells were automatically quantified by using the Olive tail moment parameter [(tail mean – head mean) × (% tail DNA/100)], where tail mean is the tail DNA intensity subtracted from background intensity, head mean is the head DNA intensity subtracted from background intensity, and % tail DNA is the fraction of DNA that has migrated from the head. The average Olive tail moments were calculated for each subject.

Statistical Analysis

Telomere length was analyzed as a continuous variable and as a categorical variable. The Wilcoxon rank sum test was used to compare the differences in telomere length between case patients and control subjects as a continuous variable. As a categorical variable, the quartile values of telomere length, according to its distribution in control subjects, were used to compare the differences in telomere length between case patients and control subjects. Additionally, telomere length was dichotomized at the 75% value in control subjects. Unconditional multiple logistic regression analysis was used to control for confounding by sex, age, or smoking status. Spearman's correlation test was used to examine the associations between telomere length and baseline genetic instability, between telomere length and γ-radiation sensitivity, and between telomere length and BPDE sensitivity, measured by the comet tail moment for case patients and control subjects separately. A never smoker was defined as an individual who had never smoked or had smoked fewer than 100 cigarettes in his or her lifetime. A former smoker was one who had a history of smoking but had stopped at least 1 year before being diagnosed with cancer (or, for control subjects, 1 year before being enrolled in the study). A current smoker was a smoker at the time of enrollment or one who had stopped smoking less than 1 year before being diagnosed with cancer (or, for control subjects, less than 1 year before being enrolled in the study). All statistical tests were two-sided.

RESULTS

Telomere length in case patients with head and neck cancer and their control subjects was measured with the Southern blot assay. We found that the mean telomere length was statistically significantly shorter in patients with head and neck cancer (6.5 kilobases [kb]) than in control subjects (7.4 kb) (difference = 0.9 kb, 95% CI = 0.5 to 1.2 kb; $P<.001$) (Table 2). Telomere length in cells from case patients with bladder, lung, and renal cell cancer and their corresponding control subjects was measured with the newly developed Q-FISH^{LSC} assay. We found that the telomere lengths (expressed as the ratio of the telomere signal of each study subject cell sample to that of the control lymphoblastoid cell line [LW5770]) were statistically significantly shorter in case patients than in control subjects. For bladder cancer, the telomere length ratio was 1.0 for case patients and 1.2 for control subjects (difference = 0.2, 95% CI of the difference = 0.1 to 0.3; $P = .005$). For lung cancer, the telomere length ratio was 1.1 for case patients and 1.4 for control subjects (difference = 0.3, 95% CI = 0.1 to 0.5; $P<.001$). For renal cell carcinoma, the telomere length ratio was 1.0 for case patients and 1.2 for control subjects (difference = 0.2, 95% CI = 0.0 to 0.4; $P = .019$) (Table 2).

Subjects were then categorized into quartiles of telomere length determined from the telomere length distribution in control subjects; the quartile of the longest telomere length (fourth quartile) was the referent. A dose–response relationship between increasing risk for head and neck cancer and the degree of telomere shortening was observed. After adjusting for age, sex, and smoking status, the adjusted odds ratios (ORs) for head and neck cancer associated with decreasing quartiles of telomere length were 0.84 (95% CI = 0.36 to 1.97), 1.77 (95% CI = 0.72 to 4.36), and 5.11 (95% CI = 1.90 to 13.77) (data not shown). Of the patients with head and neck cancer, 9% were in the fourth (longest) quartile of telomere length (Table 3), whereas 59% were in the first (shortest) quartile. Similar associations were observed for telomere lengths and patients with bladder, lung, and renal cell cancer.

Because telomere length was measured by the same Q-FISH^{LSC} assay in cells from case patients with bladder, lung, and renal cell cancer and from their control subjects and because all three cancers are tobacco-related, in subsequent analyses, we combined the results from studies of these three cancers to determine risk (Table 4). When we used the 75th percentile value of telomere length in control subjects as the cutoff between long and short telomeres in unconditional logistic regression analysis with multiple covariates to control for confounding by age, sex, and smoking status, we found that only 8% of cancer patients had longer telomeres (Table 4) and that shorter telomeres were associated with an increased risk of these cancers (OR = 4.51, 95% CI = 2.31 to 8.81). When the subjects were categorized into quartiles of telomere length, determined from the telomere length distribution in the control subjects, with the fourth (longest) quartile as the referent category, risks for these cancers differed by more than fourfold across the remaining strata. Shorter telomeres were associated with an increased risk for these three cancers in individuals younger than 55 years (OR for cancer = 24.46, 95% CI = 2.79 to 214.86), in individuals 65 years or older (OR for cancer = 8.66, 95% CI = 2.40 to 31.30), and in individuals 55–65 years old (OR for cancer = 1.50, 95% CI = 0.51 to 4.42) (Table 4). The shorter telomeres were associated with a greater risk of these cancers for females (OR = 7.62, 95% CI = 2.28 to 25.50) than for males (OR = 3.40, 95% CI = 1.51 to 7.62). When data were categorized by smoking status, shorter telomeres were associated with a higher risk of these cancers for never smokers (OR = 6.18, 95% CI = 1.72 to 22.13) and former smokers (OR = 5.25, 95% CI = 1.80 to 15.33) than for current smokers (OR = 2.77, 95% CI = 0.63 to 12.21). When data were categorized by increments of 25 pack-years of smoking intensity (which is close to the 75th percentile value for the control subjects and the median number of pack-years for the case patients), shorter telomeres were associated with a greater risk for these cancers in lighter (<25 pack-years) and never smokers (OR = 8.03, 95% CI = 3.00 to 21.54) than in heavier smokers (≥25 pack-years) (OR = 1.72, 95% CI = 0.59 to 5.00).

Table 2. Telomere length in case patients and control subjects

Cancer type	Case patients No.	Case patients Telomere length (95% CI)	Control subjects No.	Control subjects Telomere length (95% CI)	*P* value*
Head and neck†	92	6.5 (6.3 to 6.8)	92	7.4 (7.2 to 7.6)	<.001
Bladder‡	135	1.0 (1.0 to 1.1)	135	1.2 (1.1 to 1.3)	.005
Lung‡	54	1.1 (1.0 to 1.1)	54	1.4 (1.2 to 1.6)	<.001
Renal cell‡	32	1.0 (0.9 to 1.1)	32	1.2 (1.0 to 1.4)	.019

*Although the mean values of telomere length are presented, the *P* value is based on the two-sided Wilcoxon rank sum test. CI = confidence interval.

†Telomere length was measured by Southern blot analysis for case patients with head and neck cancer and control subjects and is expressed as kilobases.

‡Telomere length is presented as the telomere length ratio (expressed as the ratio of the telomere signal of each study subject cell sample to the telomere signal of the control cell line LW5770).

Table 3. Quartile distribution of telomere length in case patients and control subjects

Telomere length quartile	Case patients No. (%)	Control subjects No. (%)	P_{trend}
Head and neck cancer total*	92	92	
4th	8 (9)	22 (24)	
3rd	16 (17)	24 (26)	
2nd	14 (15)	23 (25)	
1st	54 (59)	23 (25)	
			.000
Bladder cancer total	135	135	
4th	7 (5)	33 (24)	
3rd	45 (33)	34 (25)	
2nd	41 (30)	37 (27)	
1st	42 (31)	31 (23)	
			.006
Lung cancer total	54	54	
4th	2 (4)	13 (24)	
3rd	9 (17)	14 (26)	
2nd	13 (24)	14 (26)	
1st	30 (55)	13 (24)	
			.002
Renal cell carcinoma total	32	32	
4th	1 (3)	8 (25)	
3rd	9 (28)	8 (25)	
2nd	7 (22)	8 (25)	
1st	15 (47)	8 (25)	
			.032

*Telomere length was measured by the Southern blot assay.

Table 4. Risk estimates for telomere length from combined data from the studies of bladder, lung, and renal cell cancers

Telomere length strata		No. of case patients (%)	No. of control subjects (%)	OR (95% CI)*
Overall (total)†		221	164	
	Long‡	17 (8)	42 (26)	1 (referent)
	Short	204 (92)	122 (74)	4.51 (2.31 to 8.81)§
Overall (total)*		221	164	
4th quartile		17 (8)	42 (26)	1 (referent)
3rd quartile		58 (26)	39 (24)	4.06 (1.90 to 8.68)§
2nd quartile		70 (32)	42 (26)	5.08 (2.40 to 10.75)§
1st quartile		76 (34)	41 (25)	4.41 (2.10 to 9.28)§
			(P_{trend} = .001)	
Age, y				
<55	Long	1 (2)	12 (30)	1 (referent)
	Short	50 (98)	28 (70)	24.46 (2.79 to 214.86)‖
55–64	Long	10 (17)	11 (27)	1 (referent)
	Short	50 (83)	30 (73)	1.50 (0.51 to 4.42)‖
⩾65	Long	6 (5)	19 (23)	1 (referent)
	Short	104 (95)	64 (77)	8.66 (2.40 to 31.30)‖
Sex				
Male	Long	12 (7)	24 (20)	1 (referent)
	Short	151 (93)	95 (80)	3.40 (1.51 to 7.62)¶
Female	Long	5 (9)	18 (40)	1 (referent)
	Short	53 (91)	27 (60)	7.62 (2.28 to 25.50)¶
Smoking status				
Never	Long	3 (7)	24 (30)	1 (referent)
	Short	41 (93)	55 (70)	6.18 (1.72 to 22.13)#
Former	Long	5 (5)	14 (20)	1 (referent)
	Short	105 (95)	56 (80)	5.25 (1.80 to 15.33)#
Current	Long	9 (13)	4 (27)	1 (referent)
	Short	58 (87)	11 (73)	2.77 (0.63 to 12.21)#
Cumulative smoking, pack-years				
<25	Long	5 (5)	36 (29)	1 (referent)
	Short	95 (95)	88 (71)	8.03 (3.00 to 21.54)#
⩾25	Long	12 (10)	6 (15)	1 (referent)
	Short	109 (90)	34 (85)	1.72 (0.59 to 5.00)#

*OR = odds ratio; CI = confidence interval.
†Including case patients with bladder, lung, and renal cell carcinoma.
‡Categorized by the 75th percentile value of telomere length in the control subjects.
§Adjusted by age, sex, and smoking status.
‖Adjusted by sex and smoking status.
¶Adjusted by age and smoking status.
#Adjusted by age and sex. Never smokers were included in <25 pack-years category.

We next used a stratified analysis to examine the joint effects of shorter telomere length and smoking status on the risk of tobacco-related cancer including bladder, lung, and renal cell cancers (Table 5). Subjects who were never smokers and who had longer telomeres were the referent group. After adjusting for age and sex, the risk of these cancers associated with never smoking and shorter telomeres (OR = 6.18, 95% CI = 1.72 to 22.13) and with ever smoking and longer telomeres (OR = 6.49, 95% CI = 1.54 to 27.38) were substantially lower than the risk associated with the combination of ever smoking and shorter telomeres (OR = 25.05, 95% CI = 6.91 to 90.73). Similarly, we found evidence of interaction between pack-years and telomere length. For shorter telomeres and light smoking, the OR was 8.03 (95% CI = 3.00 to 21.54), for longer telomeres and heavy smoking, the OR was 14.04 (95% CI = 3.57 to 55.16), but for shorter telomeres and heavy smoking, the OR was 29.62 (95% CI = 9.91 to 88.56), suggesting a greater-than-additive interaction.

Table 5. Cancer risk from combined data of bladder, lung, and renal cell cancer studies: joint effect of telomere length and cigarette smoking

Telomere length	Cigarette smoking status	No. of case patients	No. of control subjects	OR (95% CI)*
Long†	Never	3	24	1.00 (referent)
Short	Never	41	55	6.18 (1.72 to 22.13)
Long	Ever	14	18	6.49 (1.54 to 27.38)
Short	Ever	163	67	25.05 (6.91 to 90.73)
Long	Light‡	5	36	1.00 (referent)
Short	Light	95	88	8.03 (3.00 to 21.54)
Long	Heavy	12	6	14.04 (3.57 to 55.16)
Short	Heavy	109	34	29.62 (9.91 to 88.56)

*Adjusted by age and sex. OR = odds ratio; CI = confidence interval.
†Categorized by the 75th percentile value of telomere length in the control subjects.
‡Categorized at 25 pack-years, which is close to the 75th percentile of pack-years in control subjects and median number of pack-years in case patients.

DNA damage at baseline and induced by γ-radiation or BPDE in peripheral blood lymphocytes from study participants was quantified by the comet assay as tail moments. Results from the comet assay were available for 32 case patients with bladder cancer and 72 control subjects. We determined whether telomere length in baseline samples and mutagen-induced damage in samples after γ-radiation and BPDE exposure were correlated.

In case patients and control subjects combined, we found a statistically significant inverse correlation between telomere length and baseline and induced DNA damage, with correlation coefficients of −.28 ($P = .004$) for baseline DNA damage, −.39 ($P<.001$) for γ-radiation-induced DNA damage, and −.26 ($P = .008$) for BPDE-induced DNA damage (Table 6). These patterns were essentially the same when case patients and control subjects were analyzed separately, except that the baseline correlation in case patients was of only borderline statistical significance ($P = .090$) and, in the control subjects, no correlation was found between telomere length and BPDE-induced sensitivity ($P = .777$).

We also tested the associations between telomere length and selected demographic variables. When data from patients with bladder, lung, and renal cancer were combined in the analysis, an inverse association between telomere length and age was found that was statistically significant in case patients ($\gamma = -.14$, $P = .03$) but not in control subjects ($\gamma = -.11$, $P = .16$). No association was observed between telomere length and sex (female versus males) (1.3 versus 1.2; $P = .09$ for control subjects; and 1.0 versus 1.0; $P = .75$ for case patients). Telomere length did not differ among never smokers and former smokers in either case patients or control subjects (1.3 versus 1.2; $P = .58$ for control subjects; 1.0 versus 1.0; $P = .51$ for case patients). Similarly, the telomere length did not differ among never smokers and current smokers in either case patients or control subjects (1.3 versus 1.2; $P = .52$ for control subjects; 1.0 versus 1.1; $P = .17$ for case patients). No association was observed between telomere length and the number of pack-years smoked in either case patients or control subjects ($P = .54$ for case patients and $P = .16$ for control subjects).

Discussion

Evidence from our study supports the hypothesis that telomere dysfunction impairs chromosomal stability and is associated with an increased risk of various cancers. Our results (using two different methods to measure telomere length) demonstrated that telomere length was statistically significantly shorter in lymphocytes from case patients with head and neck cancer, bladder, lung, or renal cell carcinoma than in control subjects. We also provided evidence of an increasing risk for head and neck, lung, and renal cancer associated with progressively shorter telomeres, indicating that telomere dysfunction may be a risk factor for cancer at these sites and possibly for cancer in general.

Telomere dysfunction may be one of the molecular causes of genetic instability. Chromosomal rearrangement, generating gains and losses of segments of chromosomes, is an essential step in epithelial carcinogenesis. These extensive and complex rearrangements typically occur at an early stage when telomerase is first activated, suggesting that an early and brief period of telomere dysfunction could contribute to the complex genomic alterations observed in epithelial cancers *(11,12).* In support of this hypothesis, there is evidence that shortened telomeric DNA results in nonhomologous end joining of telomeric DNA, leading to loss of telomere function and genomic instability *(13,14).* Moreover, the combination of telomere dysfunction, rather than loss of telomerase itself, and p53 deficiency has been shown to accelerate tumorigenesis *in vivo (8,15).* Similarly, Chin et al. *(16)* found that, concurrent with severe telomere shortening and genomic instability, p53 was activated, leading to growth arrest and/or apoptosis. However, deletion of p53 attenuated the adverse cellular and organismal effects of telomere dysfunction during the earliest stages of genetic crisis. Consequently, the loss of telomere function and p53 deficiency appeared to cooperate to initiate the transformation process. Although telomerase is reactivated in most human cancers, telomere shortening and dysfunction might impair chromosomal stability early in carcinogenesis and, consequently, drive the initial carcinogenic process.

In this study, the Southern blot assay was used to measure telomere length in the head and neck cancer case–control study, but an improved Q-FISH^{LSC} assay was used in the other three case–control studies. Although Southern blot analysis of genomic DNA digested with selected restriction enzymes is the most commonly used approach, contributions of individual subtelomeric DNA fragments limit the ability of this method to provide accurate telomeric lengths. The Q-FISH^{LSC} approach has several advantages. FISH uses direct labeling of terminal telomeric repeats so that telomere length data can be accurately and quantitatively obtained. This approach correlates well with telomere restriction fragment lengths derived from Southern blot analysis *(10).* We have demonstrated statistically significant correlations between telomere length measured in the two assays, with a Spearman correlation coefficient of .47 ($P = .036$; data not shown). An important feature of the Q-FISH^{LSC} protocol is the use of an internal cell line control that provides automatic compensation for potential differences in any steps in the procedure, from fixation to hybridization and DNA staining. The control cell population also serves as an internal telomere length standard for comparing different samples with high precision. This protocol is simple, rapid, and highly reproducible.

Interestingly, we found that short telomeres appeared to play a greater role in patients younger than 55 years or older than 65 years than in patients aged 55–65 years, in women than in men, in never or former smokers than in current smokers, and in light smokers than in heavy smokers. These patterns suggest that there are genetically susceptible subgroups and that susceptibility may be less evident in the presence of constant heavy carcinogenic exposures. However, we cannot exclude the possibility that some of these findings in the subsets were caused by random variation. We have provided some preliminary validation of our telomere length assay by correlating the measurements of DNA damage obtained with the comet assay both before and after mutagen exposure. As we had predicted *a priori,* there was a negative correlation between telomere length and the level of DNA damage at baseline and after exposure to BPDE or radiation.

In support of these findings, Hanson et al. *(17)* and Kennedy and Hart *(18)* observed a statistically significant reduction in

Table 6. Correlation between telomere length and mutagen sensitivity in case patients with bladder cancer and control subjects

Treatment	Correlation coefficient* (P value)		
	Case patients and control subjects	Case patients	Control subjects
Baseline	−.28 (.004)	−.20 (.090)	−.43 (.011)
Radiation	−.39 (<.001)	−.27 (.020)	−.56 (.001)
BPDE†	−.26 (.008)	−.32 (.007)	−.05 (.777)

*Spearman's correlation test was used. All statistical tests were two-sided.
†BPDE = benzo[*a*]pyrene diol epoxide.

telomere length in patients with Fanconi anemia, who have increased susceptibility to cancer. Telomere dysfunction is also associated with advanced age when cancer incidence increases exponentially, which could be attributed in part to age-related telomere loss *(19,20)*.

Although the mechanism of how telomere dysfunction accelerates tumor onset is unclear, recent evidence has linked telomeres and DNA damage signaling or repair in cells. The DNA damage response, upon sensing an uncapped telomere or another broken DNA end, recruits repair enzymes to the DNA ends *(21,22)*. Blackburn *(21)* suggested that telomere function could be pictured as regulating and channeling the active and sensitive surveillance DNA damage response, which can detect a single DNA break in a cell and trigger an appropriate telomere-specific response to maintain telomere integrity. The usual response to the uncapping of a telomere is eliciting telomerase activity (primarily), homologous recombination, or even nonhomologous end joining. If capping fails to occur, the response of a normal cell is to exit the cell cycle or, in certain mammalian cells, to undergo apoptosis. In addition, telomeres are involved in the process of chromosomal repair, as shown by the recruitment or *de novo* synthesis of telomere repeats at double-stranded breaks and by the ability of yeast telomeres to serve as repositories of essential components of the DNA repair machinery, particularly those involved in nonhomologous end joining *(22–28)*. An association involving telomere dysfunction, chromosomal instability, impaired DNA repair, and radiosensitivity in a mammalian model system has recently been reported *(8,29–31)*. A statistically significantly inverse correlation between telomere length and chromosomal radiosensitivity in lymphocytes from patients with breast cancer and healthy control subjects was also observed *(31)*. Others *(32,33)* have reported that short telomeres are associated with hypersensitivity to ionizing radiation in mammals. In this study, our results from the phenotypic comet assay showed an inverse association between telomere length and increased levels of radiation-induced DNA damage or chemical-induced DNA damage. These findings indicate that telomere dysfunction could be involved in the development of cancer and also could have an impact on radiotherapeutic strategies for the treatment of cancer. However, in our study, telomere length was measured in a surrogate tissue, peripheral blood lymphocytes, not in cancer cells. Cancer cells have different telomere dynamics, including the reactivation of telomerase that might also modulate genomic instability.

In addition to the association with DNA damage or repair, telomere attrition may also contribute to tumorigenesis by leading to complex cytogenetic abnormalities. One possible consequence is nonhomologous end joining of telomeric DNA that may cause more telomere damage and affect genes located in sub-telomeric regions *(15)*. Artandi et al. *(8)* demonstrated that telomere attrition promotes the development of epithelial cancers in mice by a process of breakage-fusion-bridge leading to the formation of nonreciprocal translocations, a hallmark of human carcinoma, with oncogenic potential, first by carrying chimeric or deregulated oncogenes at their breakpoints, and second by altering gene dosage. Interestingly, Gonzalez-Suarez et al. *(34)* found that telomerase-deficient mice with short telomeres were resistant to skin tumorigenesis. This finding suggests that short telomeres are associated with telomerase activity because high telomerase activity results in increased propagation of cells with DNA damage. However, in telomerase-deficient mice with short telomeres, cells may not survive long enough to undergo neoplastic transformation.

In summary, we have shown, to our knowledge for the first time, that telomere length appears to be associated with increased risks for head and neck, bladder, lung, and renal cell carcinomas. Future research should focus on the associations of telomere dynamics, cell cycle checkpoints, apoptosis, the activation of telomerase, and DNA repair capacity, ultimately, with the goal of enhancing our ability to identify high-risk subgroups.

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NOTES

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