Constitutive Short Telomere Length of Chromosome 17p and 12q but not 11q and 2p Is Associated with an Increased Risk for Esophageal Cancer

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Abstract Shortened telomere length may cause chromosomal instability in Barrett's esophagus and thus promote tumorigenesis. However, whether short telomere length in all chromosomes or just some of them is associated with increased esophageal cancer (EC) risk is largely unknown. To address this question, we examined the overall and chromosome-specific telomere lengths of 17p, 12q, 2p, and 11q and assessed their associations with EC risk. In a case-control study with 94 EC cases and 94 matched controls, the overall telomere length and the chromosome-specific telomere lengths of 17p, 12q, 2p, and 11q in peripheral blood lymphocytes were determined by a real-time PCR and a modified single telomere length analysis assay, respectively. Multivariate logistic regression analysis was used to assess the association between telomere length and EC risk. Compared with controls, EC patients had significantly shorter overall telomere lengths ($P = 0.004$) and chromosome-specific telomere lengths of 17p ($P = 0.003$) and 12q ($P = 0.006$) but not of 11q ($P = 0.632$) and 2p ($P = 0.972$). Furthermore, the multivariate logistic regression analysis showed that the short overall telomere length and chromosome-specific telomere lengths of 17p and 12q were associated with a dose-dependent increase in EC risk. Our study provides the first epidemiologic evidence that short telomere length of 17p and 12q plays an important role in esophageal carcinogenesis, suggesting that short telomere length of specific chromosomes is associated with the etiology of different cancer types.

The telomere is composed of tandem DNA sequence repeats (TTAGGG in humans) that are located at the ends of chromosomes and protect them from degradation and end-to-end fusion (1, 2). In normal somatic cells, telomeres are progressively shortened with each cell division because of the end replication problem of DNA polymerase (3). Oxidative damage and loss of telomere-binding proteins can also cause telomere shortening (1, 2). This shortening can be compensated by the telomerase that is constitutively expressed in germ-line cells and in most malignant cells (4).

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Short telomere length may induce cells to undergo cell cycle arrest and apoptosis (5). In the setting of abrogated DNA damage checkpoints, critically short telomeres may cause chromosome instability and drive early carcinogenesis (6). Previous studies have reported that short overall telomeres are associated with the initiation and progression of malignancies in mouse models and epidemiologic studies (1, 2). Several recent investigations suggested that the regulation of the telomere length in mammalian cells may be chromosome specific (7, 8), leading us to hypothesize that certain specific telomeres contribute to chromosome instability and specific cancer initiation.

Esophageal cancer (EC) is one of the leading causes of cancer death worldwide (9) The molecular mechanism of this disease remains largely unknown. Meeker et al. (10) showed that telomere shortening occurs early in the etiology of esophageal carcinogenesis. Finley et al. (11) found that chromosomal instability of 17p and 11q in Barrett's esophagus is related to overall telomere length. These two findings suggest that telomere dysfunction plays a critical role in EC development. However, it remains to be seen whether and which specific chromosome telomeres are associated with the development of EC.

In this study, we used a case-control epidemiologic study to test our hypothesis that only some specific telomeres are associated with cancer risk. We measured the overall and chromosome-specific telomere lengths of 17p, 12q, 11q, and 2p in

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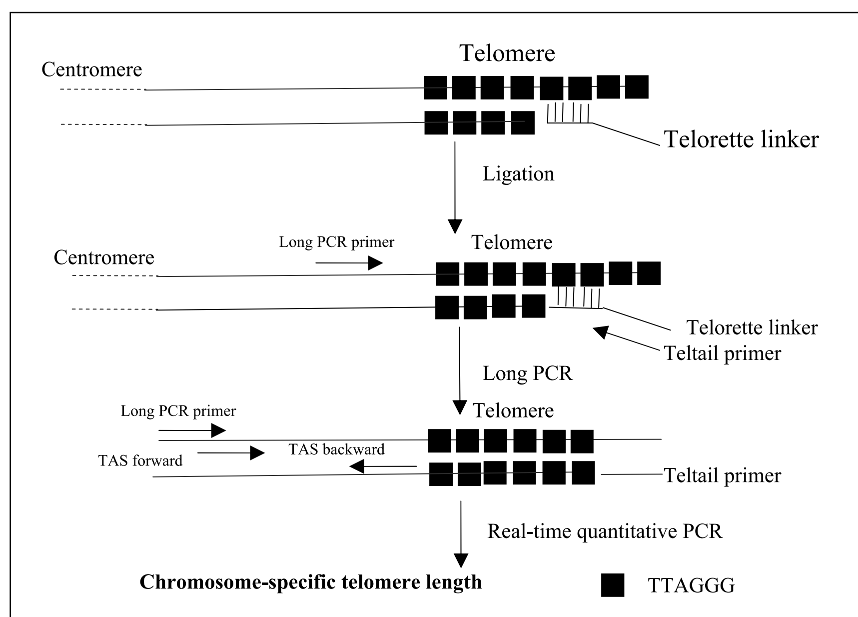

Fig. 1. Modified STELA assay procedure.

peripheral blood lymphocytes by using a real-time PCR and a modified single telomere length analysis (STELA) assay. These four chromosomes were chosen mainly due to the previous validated findings for the STELA method (8). To the best of our knowledge, this is the first epidemiologic study to investigate the role of chromosome-specific telomere length in cancer etiology.

Materials and Methods

Study population and epidemiologic data

In our ongoing EC case-control study (started from November 2004), patients were recruited from The University of Texas M. D. Anderson Cancer Center. Eligible cases were histologically confirmed, previously untreated, and diagnosed within 1 y of enrollment. There was no restriction on age, sex, ethnicity, or tumor stage. Healthy controls without a previous cancer history (except nonmelanoma skin cancer) were recruited from the Kelsey-Seybold Clinic, the largest multispecialty physician group in the Houston metropolitan area. Because the assays described in this study, particularly chromosome-specific telomere length assays, are time consuming and costly, and because analyses of phenotypic assays typically have high statistical power, we only selected 94 consecutively recruited Caucasian cases and 94 age-, gender-, and ethnicity-matched controls. The distributions of epidemiologic variables of these selected cases and controls were not significantly different from those of the whole case and control population. The majority of cases (85.1%) had adenocarcinoma with 13.8% of the cases diagnosed with squamous cell carcinoma. The distributions of tumor stages were as follows: stage I, 9.6%; stage II, 25.5%; stage III, 34%; and stage IV, 26.6%. The response rates for cases and controls were 91% and 76.7%, respectively.

Information on demographics, smoking history, and family history of cancer was collected using a standardized questionnaire. Smoking status and pack-years have been previously defined and described (12). We collected 40 mL of blood in coded heparinized tubes. This study was approved by all relevant review boards, and written informed consent was obtained from all participants.

Overall telomere length assessment by real-time PCR

High-quality genomic DNA was extracted from participants' whole blood using the QIAamp Maxi DNA kit (Qiagen) according to the manufacturer's protocol. The relative overall telomere length was measured by using a modified version of the real-time quantitative PCR as described by Cawthon (13). Briefly, there are two main steps involved in the relative quantification of telomere lengths. First, the ratio of the telomere repeat copy number and the single gene (human globulin) copy number is determined for each sample using standard curves. The derived ratio is proportional to the overall telomere length. For the second step, the ratio for each sample was then normalized to a calibrator DNA sample to standardize between different runs.

The PCR (15 μL) for the telomere amplification consisted of 1× SYBR Green Mastermix (Applied Biosystems), 200 nmol/L Tel-1, 200 nmol/L Tel-2, and 5 ng of genomic DNA. The PCR for human globulin amplification consisted of 1× SYBR Green Mastermix, 200 nmol/L Hgb-1, 200 nmol/L Hgb-2, and 5 ng of genomic DNA. The thermal cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 56°C (for telomere amplification) or 58°C (for Hgb amplification) for 1 min. The telomere and Hgb PCRs were done on separate 384-well plates, with the same samples in the same well positions.

In each run, negative and positive controls, a calibrator DNA, and a standard curve were included. The positive controls contained a telomere of 1.2 kb and a telomere of 3.9 kb from a commercial telomere length assay kit (Roche Applied Science). For each standard curve, one reference DNA sample (the same DNA sample for all runs) was diluted by using a 2-fold increment per dilution to produce a six-point standard curve between 20 and 0.625 ng of DNA in each reaction. The R^2 for each standard curve was ≥0.99, with acceptable SDs set at 0.25 (for the C_t values). If the result was found to be out of the acceptable range, the sample was repeated. For testing the interassay variation, two samples with relatively low and high telomere lengths were tested using three different runs.

Chromosome-specific telomere length assessment by STELA assay

The chromosome-specific telomere lengths of 17p, 12q, 11q, and 2p were determined by a STELA method as previously described (8, 14)

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with some modifications. The assay procedure was sketched in Fig. 1. Briefly, in the first step, genomic DNA from each sample was ligated with telorette linkers at 35°C for 12 h in a final reaction volume of 20 μL containing 50 ng of high-quality genomic DNA, 240 pmol linker mix (40 pmol for each), and 1 unit of T4 DNA ligase (Amersham Biosciences) in 1× manufacturer's ligation buffer. Ligated genomic DNA was purified using the DNA Cleanup kit (Promega). The concentration of the purified DNA was determined using the PicoGreen dsDNA assay kit (Invitrogen).

In the second step, ligated genomic DNA was diluted in water to 0.5 ng/μL. Then, the long PCR was carried out for each ligated DNA sample in a volume of 15 μL containing 1 ng of ligated DNA, 4.5 pmol of each telomere-adjacent (17p, 12q, 11q, and 2p) primer, 4.5 pmol of teltail primer, 5.4 nmol deoxynucleotide triphosphate, and 1.5 units of enzyme mix (Roche Applied Science) in 1× manufacturer's PCR buffer 1. The PCR conditions were as follows: 17p, 94°C for 3 min and 25 cycles at 94°C for 15 s, 59°C for 30 s, and 68°C for 12 min; 12q, 94°C for 3 min and 28 cycles at 94°C for 15 s, 62°C for 30 s, and 68°C for 9 min; 11q, 94°C for 3 min and 28 cycles at 94°C for 15 s, 66°C for 30 s, and 68°C for 9 min; 2p, 94°C for 3 min and 28 cycles at 94°C for 15 s, 65°C for 30 s, and 68°C for 9 min. All long PCR products were diluted in water at a 1:320 ratio for use in real-time PCR.

In the final step, the real-time PCR telomere length assay was modified to assess chromosome-specific telomere length using the chromosome-specific telomere-adjacent sequence as a replacement for a single-copy gene. The PCR for the telomere amplification remained the same as in the overall telomere length assay, except the 5.5 μL diluted long PCR product was used as a template instead of the genomic DNA, and 200 nmol/L of telomere-adjacent sequence forward and backward primers each were included in the PCR. Laboratory personnel were blinded to the case-control status of each sample during all the experiments.

Statistical analysis

All statistical analyses were done using the STATA 8.0 statistical software (STATA Corp.). The difference in the distribution of host characteristics between the cases and the controls was evaluated by the Pearson χ^2 test for categorical variables (gender and smoking status) and the Student's t test for continuous variables (age, pack-years, and telomere length). Telomere lengths were also analyzed as categorical variables by setting a cutoff point at the median or tertile values in the controls. The association between EC risk and telomere length was estimated by an unconditional multivariate logistic regression to determine the adjusted odds ratio (aOR) and 95% confidence interval (95% CI), adjusting for age, sex, smoking status, drinking status, education, and physical activity. All statistical tests were two sided, and the level of statistical significance was set at $P < 0.05$.

Table 1. Distribution of selected characteristics of EC cases and controls

Variable	Cases (*n* = 94)	Controls (*n* = 94)	*P*
Sex, *n* (%)			
Male	88 (94)	88 (94)	
Female	6 (6)	6 (6)	1.00
Mean age, y (SD)	62.7 (9.2)	63.0 (9.1)	0.81
Smoking status,* *n* (%)			
Never	16 (17)	33 (35)	
Ever	76 (83)	61 (65)	0.01
Pack-years, mean (SD)†	36.2 (25.8)	36.0 (24.2)	0.97

*Smoking status was unavailable for two patients.
†Ever smokers only.

Table 2. Difference in overall and chromosome-specific telomere lengths between EC cases and controls

Telomere length	Cases *n*,* mean (SD)†	Controls *n*,* mean (SD)†	*P*
Overall	94, 1.18 (0.40)	92, 1.36 (0.43)	0.004
17p	86, 1.14 (0.39)	84, 1.46 (0.92)	0.003
12q	86, 1.41 (0.97)	87, 2.07 (2.01)	0.006
2p	86, 1.17 (0.58)	82, 1.18 (0.56)	0.972
11q	84, 0.77 (0.78)	84, 0.71 (0.65)	0.632

*Totals may not add to 94 because of insufficient samples or assay failure.
†The telomere length was measured as a relative value, T/S ratio.

Results

The oligonucleotide sequences of all primers and linkers used in this analysis are fully described in Supplementary Table S1. The characteristics of the cases and controls are summarized in Table 1. The cases had more ever smokers than the controls (83% versus 65%, $P = 0.01$), but the pack-years for ever smokers were similar between the cases and controls ($P = 0.97$).

Table 2 shows the overall and chromosome-specific telomere lengths. The mean interassay coefficients of variation for overall, 17p, 12q, 11q, and 2p telomere length were 2.6%, 5.0%, 7.4%, 5.1%, and 5.7%, respectively (results not shown). The overall telomere lengths were significantly shorter in cases than in controls (mean ± SD, 1.18 ± 0.40 versus 1.36 ± 0.43; $P = 0.004$). The same was the case for 17p (1.14 ± 0.39 versus 1.46 ± 0.92; $P = 0.003$) and 12q telomere length (1.41 ± 0.97 versus 2.07 ± 2.01; $P = 0.006$) but not for 11q (0.77 ± 0.78 versus 0.71 ± 0.65; $P = 0.632$) and 2p telomere length (1.17 ± 0.58 versus 1.18 ± 0.56; $P = 0.972$). A significant inverse association was found between age and overall telomere length in the controls ($r = -0.289$, $P = 0.005$), and the inverse association was weaker in the cases ($r = -0.173$, $P = 0.096$; Fig. 2A and B). No significant inverse association was found for any of the four specific telomeres with age in controls (data not shown), but the 17p ($r = -0.239$, $P = 0.027$) and 2p ($r = -0.246$, $P = 0.022$) telomere length was inversely associated with age in the cases (Fig. 2C and D). We did not find a significant correlation between overall telomere length and any of the four specific telomere lengths in either EC cases or controls (data not shown).

Table 3 shows the associations between telomere lengths and EC risk. Using the median value of the telomere lengths in the controls as the cutoff point, we found that individuals with short overall telomere lengths had a significantly increased EC risk (aOR, 2.52; 95% CI, 1.29-4.94) after adjustment for age, sex, smoking status, drinking status, education, and physical activity. Short 17p telomere was associated with a

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borderline significantly increased EC risk (aOR, 1.90; 95% CI, 0.96-3.73). Short 12q telomere also seemed to increase EC risk (aOR, 1.63; 95% CI, 0.85-3.12) but not short 2p (aOR, 1.30; 95% CI, 0.67-2.51) or 11q telomeres (aOR, 0.91; 95% CI, 0.47-1.75).

A significant dose-response relationship was observed between EC risk and overall telomere length (*P* for trend = 0.03), 17p telomere length (*P* for trend = 0.03), and 12q telomere length (*P* for trend = 0.02) in tertile analyses (Table 3). For example, compared with individuals in the longest tertile of 17p telomere lengths, individuals in the medium and shortest tertiles had a significantly higher EC risk, with aORs of 2.06 (95% CI, 0.83-5.13) and 2.83 (95% CI, 1.15-6.95), respectively.

We then did a stratified analysis to determine the joint effects between telomere lengths and smoking on EC risk. We found significant joint effects between smoking and overall (Fig. 3A) as well as 17p (Fig. 3B) and 12q telomere length (Fig. 3C). For example, when never smokers with long overall telomere lengths were used as the reference group, the aOR for never smokers with short overall telomere length, ever smokers with long overall telomere lengths, and ever smokers with short overall telomere length were 5.39, 5.88, and 11.78, respectively. The interaction terms were not significant between smoking and telomere lengths (*P* for interaction = 0.20, 0.60, and 0.31 for overall, 17p, and 12q telomeres, respectively) (results not shown).

Discussion

There are three major observations from this study. First, short overall telomere length was associated with an increasing EC risk. Second, short telomere length of 17p and 12q was associated with an increased EC risk. Third, we found a significant joint effect between smoking and overall or chromosome-specific 17p and 12q telomere length in elevating EC risk. This is the first epidemiologic study to evaluate chromosome-specific telomere length as a potential biomarker for risk prediction in any cancer.

Our findings agree with a previous report by Risques et al. (15) showing that overall telomere length of blood leukocyte is associated with EC risk in patients with Barrett's esophagus. Several previous epidemiologic studies in other cancers (1, 2, 16) showed that constitutive short overall telomere length in peripheral blood lymphocytes is a strong cancer susceptibility marker, consistent with previous laboratory results (10, 17) that telomere dysfunction is an early alteration acquired in the multistep process of malignant transformation. Biologically, shortened telomeric DNA results in nonhomologous end joining, which in turn leads to genomic instability (18). In addition, cells with telomere dysfunction are also associated with decreased DNA repair capacity and complex cytogenetic abnormalities (19).

Although it is increasingly clear that short overall telomere length contributes to tumorigenesis, no epidemiologic reports have addressed whether all telomeres or only a few specific

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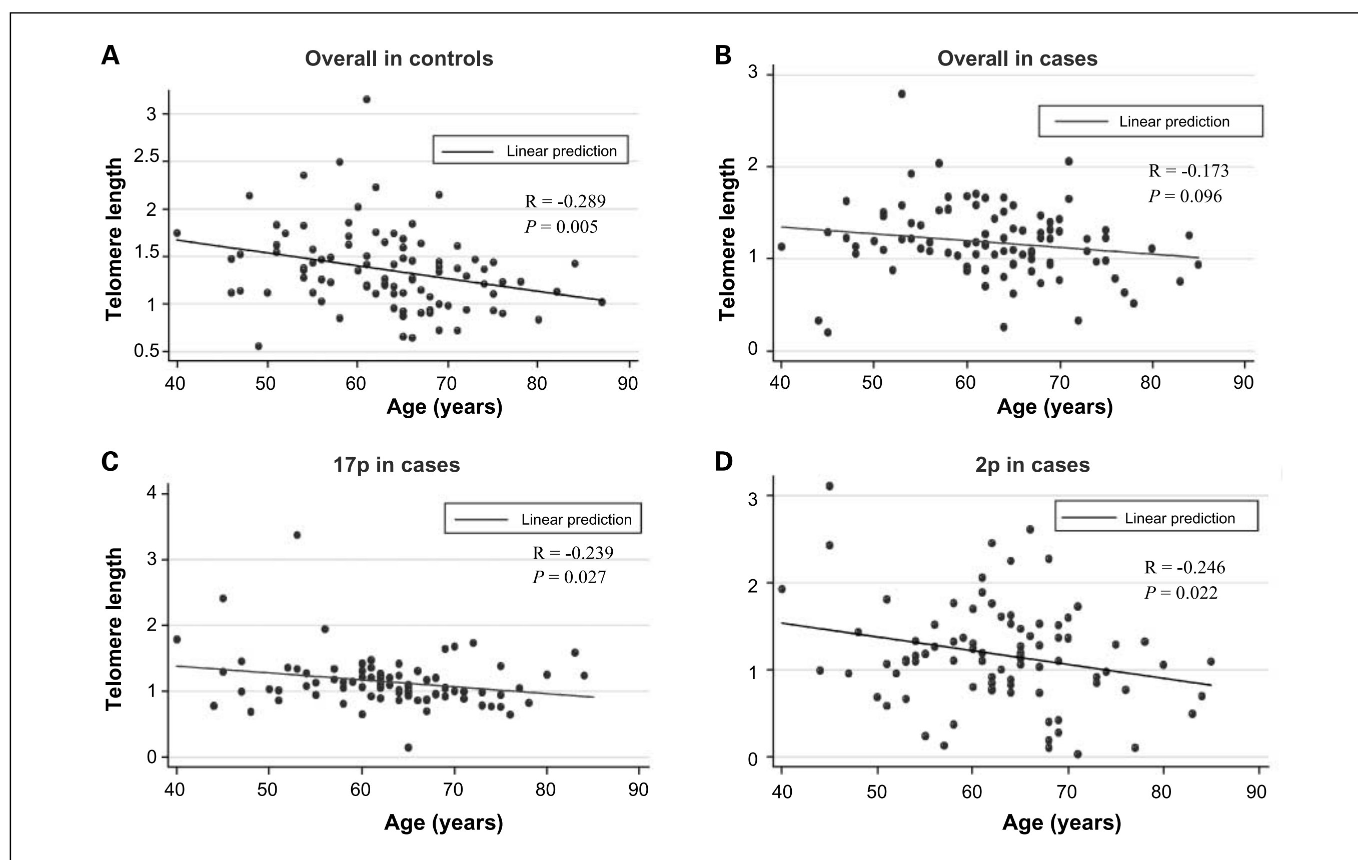

Fig. 2. Correlation between age and overall telomere length in controls (*A*) and EC cases (*B*) and between age and 17p (*C*) and 2p (*D*) telomere length in cases.

Table 3. EC risk as estimated by telomere length

Telomere length	Cases, *n* (%)*	Controls, *n* (%)*	aOR† (95% CI)
Overall			
By median			
≥1.315	28 (30)	46 (50)	Reference
<1.315	66 (70)	46 (50)	2.52 (1.29-4.94)
By tertile			
3rd	21 (22)	30 (33)	Reference
2nd	28 (30)	32 (34)	1.10 (0.47-2.59)
1st	45 (48)	30 (33)	2.38 (1.04-5.46)
P for trend			0.03
17p			
By median			
≥1.218	26 (30)	42 (50)	Reference
<1.218	60 (70)	42 (50)	1.90 (0.96-3.73)
By tertile			
3rd	12 (14)	28 (33)	Reference
2nd	32 (37)	28 (33)	2.06 (0.83-5.13)
1st	42 (49)	28 (33)	2.83 (1.15-6.95)
P for trend			0.03
12q			
By median			
≥1.492	35 (49)	44 (51)	Reference
<1.492	51 (51)	43 (49)	1.63 (0.85-3.12)
By tertile			
3rd	7 (22)	29 (33)	Reference
2nd	44 (30)	29 (33)	6.28 (2.25-17.50)
1st	35 (48)	29 (33)	4.64 (1.66-12.95)
P for trend			0.02
2p			
By median			
≥1.155	42 (49)	41 (50)	Reference
<1.155	44 (51)	41 (50)	1.30 (0.67-2.51)
By tertile			
3rd	23 (22)	27 (33)	Reference
2nd	36 (30)	28 (34)	1.75 (0.77-3.95)
1st	27 (48)	27 (33)	1.46 (0.63-3.39)
P for trend			0.38
11q			
By median			
≥0.610	43 (51)	42 (50)	Reference
<0.610	41 (49)	42 (50)	0.91 (0.47-1.75)
By tertile			
3rd	32 (38)	28 (33)	Reference
2nd	35 (42)	28 (33)	1.14 (0.53-2.50)
1st	17 (20)	28 (33)	0.53 (0.22-1.25)
P for trend			0.17

*Totals may not add to 94 because of insufficient DNA samples or assay failure.

†Adjusted by age, sex, smoking, drinking status, education, and physical activity.

telomeres contribute to tumorigenesis in a specific cancer type. An early review (20) has suggested that there is a chromosome-specific pattern of telomere lengths. Moreover, Graakjaer et al. (7) showed that this pattern is partly inherited in humans and maintained throughout life. der-Sarkissian et al. (21) suggested that loss of telomere function occurs preferentially on chromosomes with critically short telomeres in transformed embryonic kidney epithelial cells. A mouse model of telomerase knockout revealed that the shortest telomeres, not the overall telomeres, contribute to telomere dysfunction and limit cellular survival in the absence of telomerase (22). In addition, Leach et al. (23) observed that human chromosomes with shorter telomeres have a higher frequency of acquired somatic cell aneuploidy in cultured lymphocytes. Collectively, these reports strongly suggest that only shorter telomeres are possibly involved in tumorigenesis. In the present study, our findings provided the first epidemiologic evidence that short telomere length of specific chromosomes, such as 17p and 12q, is associated with an increased EC risk, suggesting that some specific telomeres might play a more prominent role in the development of EC than other chromosome telomeres. Therefore, the identification and measurement of chromosome-specific telomere length could be useful for the prediction of EC risk.

Martens et al. (24) observed that chromosome 17p tended to be one of the shortest chromosomes. They also suggested that the loss of heterozygosity on 17p may be caused by the shortened telomere of 17p. Accordingly, high frequencies of loss of heterozygosity on chromosome 17p have been reported in many types of cancer, including EC (25-27). The *p53* gene and other possible tumor suppressor genes are located on 17p, and as such, it is conceivable to consider that the telomere of 17p may somehow be implicated in tumorigenesis (28). In addition, Finley et al. (11) observed that the loss of 17p was the most common event in Barrett's esophagus and that this loss was significantly associated with an overall telomere length shortening ($r = 0.55$; $P < 0.0001$). These reports have provided a strong concordance for our finding that the short 17p telomere length is a critical risk factor for EC development. Our data also indicate that the short telomere length of 12q is also associated with an increased EC risk. Previous comparative genomic hybridization studies of EC have also shown that chromosome aberrations were frequently found on 17p and 12q (29, 30). In a human tumor cell line, Sabatier et al. (31) showed that the loss of a chromosome-specific telomere length could result in the instability of multiple chromosomes. Therefore, it is possible that the telomere dysfunction of 17p and 12q can result in the chromosome instability of not only these chromosomes but others as well. Future research should focus on uncovering the biological mechanisms underlining specific chromosome dysfunction.

Telomere length is a biomarker of biological age, and age-dependent shortening of telomeres in most somatic cells impairs cellular function and viability (32). Therefore, we evaluated the correlation between age and overall or chromosome-specific telomere length. Our results support earlier findings by Valdes et al. (33) and O'Sullivan et al. (34) that overall telomere length decreases linearly with age. The inverse relation between age and chromosome-specific telomere lengths was only found in cases for 17p and 2p but not in controls. In addition, we did not find correlations between overall and chromosome-specific telomere length of 17p, 12q, 11q, and 2p. There are two possible explanations for these results. One explanation could be that chromosome-specific telomere lengths seem to vary considerably more in cases and controls than overall telomere

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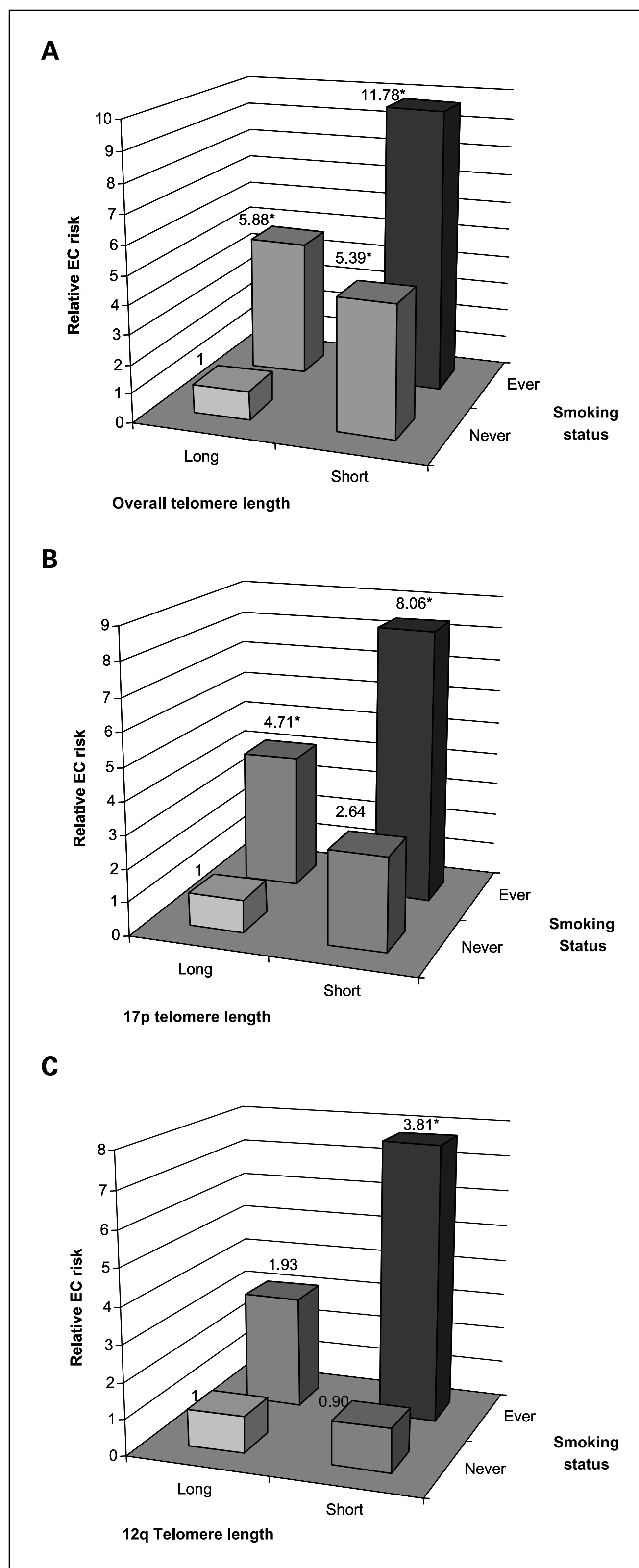

Fig. 3. Joint effect on EC risk between smoking status and overall telomere length (*A*) or chromosome-specific telomere length of 17p (*B*) and 12q (*C*). Participants were dichotomized into two groups using the median values in the controls for overall and chromosome-specific telomere lengths. The cutoff points for overall, 17p, and 12q telomere lengths were 1.315, 1.218, and 1.492, respectively. ORs were adjusted by age, sex, drinking status, education, and physical activity. *, $P < 0.05$.

length. Another possibility is that the limited sample size and age ranges reduce the power to detect an association of such a small magnitude in these studies. Future studies of larger sample sizes are needed to assess the correlation of overall telomere and chromosome-specific telomere lengths as well as the association of chromosome-specific telomere lengths and age.

Cigarette smoking is an important risk factor for EC (9). Consistent with our previous studies (1, 2), we found a significant joint effect between overall telomere length and smoking in elevating cancer risk. In addition, a joint effect between smoking and short telomere length of 17p and 12q length was also observed. These observations support the notion of gene-environment interaction in cancer etiology.

We used peripheral blood lymphocyte as a surrogate tissue. Friedrich et al. (35) measured the telomere length in three unrelated tissues and found a significant linear correlation in each pair of the two different tissues donated by the same participant. Martens et al. (24) also showed that chromosomes from different tissues donated by the same individual have a similar pattern of telomere lengths. These results support that easily accessible tissues such as blood could serve as surrogates for target tissues when measuring the relative telomere length.

In previous studies (1, 2), the quantitative fluorescence *in situ* hybridization–based approaches were commonly used to determine chromosome-specific telomere length. However, metaphase chromosomes are required for that technique, which limits researchers to only analyze proliferating cells and produce small data sets. Baird et al. (14) developed the DNA-based STELA assay. However, this approach still uses the traditional, time-consuming Southern blot to determine chromosome-specific telomere lengths after long PCR. This disadvantage may limit its application in large epidemiologic studies. Recently, Cawthon (13) described a simple real-time quantitative PCR-based method for assessing overall telomere length. Therefore, we modified the final step in the STELA assay by using real-time quantitative PCR to assess chromosome-specific telomere length instead of Southern blot. Our own internal validation results have shown that the modified STELA assay is reliable and simple. Nonetheless, only four autosomal chromosomal telomere primer pairs have been verified for the STELA assay. When more primer pairs are designed, a more comprehensive investigation of chromosome-specific telomere patterns will be possible.

In summary, our findings showed that short telomere length of 17p and 12q plays a more prominent role in the etiology of EC than 11q and 2p telomeres. It would be interesting to test in other cancer types whether different chromosome telomeres exhibit different cancer risk–modifying effect, which would support the novel hypothesis that in addition to overall short telomere length contributing to generally increased cancer risks, chromosome-specific telomeres may be involved in specific cancer etiology. Future larger studies and more comprehensive profiling of chromosome-specific telomeres are warranted to confirm and extend our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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