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Strong association between long and heterogeneous telomere length in blood lymphocytes and bladder cancer risk in Egyptian

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Abstract

Although it is widely recognized that telomere dysfunction plays an important role in cancer, the relationship between telomere function and bladder cancer risk is not well defined. In a case–control study of bladder cancer in Egypt, we examined relationships between two telomere features and bladder cancer risk. Telomere fluorescent *in situ* hybridization was used to measure telomere features using short-term cultured blood lymphocytes. Logistic regression was used to estimate the strength of association between telomere features and the risk of urothelial carcinoma of the bladder. High telomere length variation (TLV) across all chromosomal ends was significantly associated with an increased risk of bladder cancer [adjusted odds ratios (OR) = 2.22, 95% confidence interval (CI) = 1.48–3.35], as was long average telomere length (OR = 3.19, 95% CI = 2.07, 4.91). Further, TLV and average telomere length jointly affected bladder cancer risk: when comparing individuals with long telomere length and high TLV to those with short telomere length and low TLV, the adjusted OR was 14.68 (95% CI: 6.74–31.98). These associations were stronger among individuals who are 60 years of age or younger. In summary, long and heterogeneous telomere length in blood lymphocytes was strongly associated with an increased bladder cancer risk in Egyptian and the association was modulated by age.

Introduction

Telomeres, the nucleoprotein complexes at the end of eukaryotic chromosomes, are specialized structures that protect chromosome ends (1). Telomeres are composed of TTAGGG repeats and a specific associated protein complex termed shelterin (2), which regulates telomere protection and length. Telomerase is the key telomere maintenance enzyme. Most adult human cells have limited amount of telomerase so that telomere loss still occurs (3) and successive cell divisions lead to progressive telomere attrition due to the end-replication problems (4–6). Continued proliferation of cells with very short telomeres results in loss of telomere protection that ultimately leads to chromosomal instability (7–9). Since telomere shortening limits the lifespan of cells and prevents the onset of immortality, this mechanism has long been regarded as an important tumor-suppressive pathway (10). However, almost all cancer cells have found ways to escape from the normal replicative limitation through maintaining their telomeres, either by upregulation of telomerase (11) or by an alternative lengthening of telomeres (ALT) mechanism (12,13).

Although it is widely recognized that dysfunction in telomere maintenance plays an important role in human carcinogenesis,

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Abbreviations

ALT	alternative lengthening of telomeres
BMI	body mass index
CV	coefficient of variation
TLV	telomere length variation
TL	telomere length

the relationship between telomere protection and the risk of developing bladder cancer has not been well studied. Average telomere length as a biomarker of bladder cancer risk has been examined in three case–control studies (14–16). These studies reported that shorter average telomere length in blood leucocytes was associated with an increased risk of bladder cancer. For patients with the shortest telomere lengths compared to those with the longest, Broberg *et al.* (15) found a significant 4.5-fold increase in bladder cancer risk. Russo *et al.* (17) reported that shorter leukocyte telomere length is independently associated with poor survival in bladder cancer patients. However, to the best of our knowledge, the relationship between telomere length and bladder cancer risk in Egypt population has not been reported and there are no studies that examined the other telomere features in relation to bladder cancer risk.

Average telomere length has been commonly used to assess the telomere function and cancer risk. However, average telomere length only represents an estimate of the abundance of telomere sequences in a given cell and does not provide any information on how the telomere sequences are distributed across all the chromosomal ends. Studies of human cancer cell lines have shown that the shortest telomeres, not average telomere length, drove chromosome instability in cancer cells. Chromosomal arms possessing the shortest telomeres were more often found in telomere fusions, leading to chromosomal abnormality (18–20). Our recent data showed that telomerase, the key telomere maintenance enzyme, not only elongates short telomeres, but also shortens excessively long telomeres in normal and cancerous human cells (21). These dual functions of telomerase indicate that keeping an optimal telomere length for each chromosomal end is a critical aspect of telomere maintenance and may be important for the protection of the genome. This notion is further supported by results from studying telomerase negative and ALT positive cancers. Telomerase negative and ALT positive cancer cells, i.e. osteosarcomas, are characterized by long average telomere length and striking telomere length heterogeneity (22,23). Despite having long average telomere length, these types of cancers typically exhibit increased chromosome instability and poor clinical outcome (22,24,25). Together, these previous data lead us to hypothesize that the variation in telomere lengths across all the chromosomal ends represents an important measure of telomere function and maybe a useful cancer risk biomarker. In the present study, in addition to average telomere length, we examined association between telomere length variation (TLV) across all chromosomal ends and bladder cancer risk, and found that TLV in combination with average telomere length in blood lymphocytes are significant and strong bladder cancer risk predictors in Egyptian.

Materials and methods

Study population

The study population accrual and eligibility criteria were described previously (26). In brief, bladder cancer cases were recruited from two referral cancer centers in Egypt: the National Cancer Institute in Cairo and the South Egypt Cancer Institute in Assiut. These institutions are the main cancer care centers in their regions. Eligible cases were adults between 19 and 80 years of age, self-identified as being able to participate in an interview, and diagnosed within 12 months with presumed bladder cancer. Patients who had a prior history of other cancers were excluded. For each case, the pathology report and available H&E slides prepared from the surgical or biopsy specimen of urinary bladder tissue were reviewed by either one of the two study pathologists (B.K. and I.G.) and reported it as (i) urothelial carcinoma (UC), (ii) squamous cell carcinoma (SCC), (iii) adenocarcinoma or (iv) other, including undifferentiated carcinomas. Carcinoma that metastasized to the bladder was excluded. Patients with benign bladder lesions were excluded (~7% enrolled patients). Bladder cancer patients who received systemic treatment were also excluded. This report includes only urothelial carcinoma cases (N = 224) and control subjects (N = 254) enrolled between January 2010 and April 2012.

Non-cancer controls were randomly selected from the general population to frequency-match the cumulative group of cases on gender and age (5-year interval). All controls fulfilled the following eligibility criteria: (i) no known diagnosis of any cancer; (ii) between ages 19 and 80 and (iii) self-identified as being able to participate in an interview. Using a portable ultrasound machine, the physician accompanying the recruitment team conducted an abdominal ultrasound examination to rule out asymptomatic abdominal mass. The interview and phlebotomy were conducted at the participants' home.

After explaining the study and obtaining the consent, trained interviewers administered to both cases and controls a structured questionnaire, assessing sociodemographic characteristics including current residence and birth governorate, prior medical history with emphasis on schistosomiasis or other urinary tract infection, cigarette and water pipe smoking status and history, and reproductive history (for women). Histories of exposure to environmental tobacco smoke at home and outside the home were also recorded.

The study was approved by the Institutional Review Boards of Georgetown University, the two collaborating cancer centers in Egypt and the National Scientific and Research Ethical Committee at the Egyptian Ministry of Health and Population. All participants signed an informed consent and donated a blood sample. Blood was obtained by trained phlebotomists in heparinized tubes and blood samples from cases were collected before any systemic treatments were given to the patients. The time between blood collection and bladder cancer diagnosis ranges from right before the first cystoscopy for suspected bladder lesions (before diagnosis) to 12 months after the diagnosis. The blood samples were drawn at the time when patients visited health care facilities (cases) or when recruiter visited homes of participants (controls).

Chromosome preparation from short-term culture of blood lymphocyte

Blood lymphocyte cultures were set up within 24 h after the blood draw, following the protocol as described previously (27). Briefly, 1 ml of fresh whole blood was added to 9 ml of RPMI-1640 medium, supplemented with 15% fetal bovine serum, 1.5% of phytohemagglutinin and 100 unites/ml each of penicillin and streptomycin. The blood lymphocytes were cultured at 37°C for 4 days (92–96 h) and on the day of harvesting, colcemid (0.2 μg/ml) was added to the culture and incubated at 37°C for additional 1 h. The cells were then treated in a hypotonic solution (0.06 M KCl) and fixed in the fixative (three parts of methanol with one part of acetic acid). The fixed cells were kept at –20°C for future assays.

Measurement of telomere features

T lymphocytes that responded to phytohemagglutinin stimulation during blood culture (metaphase cells) were used for the telomere measurement in this study. Telomere length at each of the chromosomal ends was measured by telomere quantitative fluorescent *in situ* hybridization (TQ-FISH) as described previously (28). Briefly, chromosome preparations were dropped onto clean microscopic slides and hybridized with 15 μl of hybridization mixture consisting of 0.3 μg/ml Cy3-labeled telomere-specific peptide nucleic acid probe, 1 μl of cocktails of fluorescein isothiocyanate-labeled centromeric peptide nucleic acid probes specific for chromosomes 2, 4, 8, 9, 13, 15, 18, 20 and 21, and 20 μg/ml of Cy3-labeled centromeric peptide nucleic acid probes specific for chromosome X (Biomarkers, Rockville, MD), in 50% formamide, 10 mM Tris–HCl, pH 7.5 and 5% blocking agent. Slides were denatured at 75°C for 5 min and then hybridized at 30°C for 3 h. After hybridization, the slides were sequentially washed 10 min each at 42°C, once in 1 × SSC, once in 0.5 × SSC and once in 0.1 × SSC. The slides

were then mounted in antifade mounting medium containing 300 ng/ml 4′-6-diamidino-2-phenylindole (DAPI).

After TQ-FISH, cells were analyzed using an epifluorescence microscope equipped with a charge-coupled device camera. Metaphase cells were captured with exposure times of 0.15, 0.25 and 0.05 s for Cy3, fluorescein isothiocyanate and DAPI signals, respectively. Digitized metaphase images were analyzed using the Isis software (MetaSystems Inc., Boston, MA), which permits simultaneous measurement of telomere signals of 92 chromosomal ends after karyotyping. Telomere fluorescent intensity units were recorded as an indirect measurement of telomere length. For each study subject, 25–30 metaphase cells were randomly selected from one or two slides and analyzed. The metaphase cell selection process is as following: up to 500 metaphase cells were first selected automatically using a predefined algorism by the image system that has the automatic scanning capability. Then 30 metaphases were manually selected based on quality of the spreads and random distribution among the 500 cells.

Several quality control steps were implemented in telomere measurement. Laboratory personnel were blinded to the case–control status of the subjects. All new lots of reagents were tested to ensure optimal hybridization. A control slide containing cells with known telomere length was included in each batch of TQ-FISH to monitor the quality of the hybridization efficiency. Case and control samples were analyzed together in each batch and a total of 18 batches were run for the whole case–control set. Analysis of control slides from 18 batches showed that the coefficient of variation of average TL and TLV were 10.98 and 12.79%, respectively.

Variable definition and statistical analysis

Average telomere length was the average telomere fluorescent intensity units per telomere. TLV was defined as the CV of all measured telomere lengths. Tobacco use was categorized as 'never users', 'waterpipe only', 'cigarette only' and 'both waterpipe and cigarette'. Participants who had smoked less than 100 cigarettes in their lifetime and had never smoked a waterpipe were classified as 'never users'; those who smoked less than 100 cigarettes in their lifetime but reported smoking waterpipe were classified as 'waterpipe only' users; 'cigarette only' users were those who had never smoked waterpipe but had smoked at least 100 cigarettes in their lifetime and 'both waterpipe and cigarette' users were those reported smoking at least 100 cigarettes in their lifetime and also used waterpipe.

Student's *t* test and Chi-square test were used to compare continuous variables and categorical variables between cases and controls, respectively. Unconditional logistic regression was used to assess the risk of bladder cancer in relation to the telomere variables. The analyses were stratified by age since age has been identified in other studies as an important risk factor associated with telomere and cancer risk. All models were adjusted for the matching factors—age and gender. In addition, multivariate models were adjusted for body mass index (BMI), education (none, literacy classes/primary school, preparatory/high/technical school or college/university) and tobacco use (as defined above). Interactions of age with telomere variables were assessed by including the relevant product terms in the logistic model. *P* values were two-sided and considered statistically significant if $P < 0.05$. All analyses were performed using SAS software, version 9.3 (SAS Institute, Cary, NC).

Results

Characteristics of study population

Table 1 summarizes selected demographic characteristics of the case and control subjects. There were no significant differences in the distributions of age, gender and marital status between bladder cancer patients and control subjects. The bladder cancer cases were significantly more likely than the controls to be smokers ($P < 0.001$), with 43.8% of cases being current smokers compared to only 18.2% of controls. The bladder cancer cases also had lower education levels ($P < 0.001$) and lower mean BMI ($P = 0.002$) compared with controls. Sixty-eight percent of the cases had muscle invasive disease. There were no significant differences for the means of the average telomere length and TLV between muscle invasive and non-invasive cases.

Associations between telomere features and host factors

Average telomere length was moderately and inversely correlated with TLV in cases [Pearson correlation coefficient (r) = –0.51, $P < 0.001$) and in controls ($r = -0.38$, $P < 0.001$). TLV was modestly correlated with age in cases ($r = 0.14$, $P = 0.04$) and in controls ($r = 0.14$, $P = 0.02$). Average telomere length was significantly and inversely correlated with age in cases ($r = -0.22$, $P = 0.001$), but not in controls ($r = -0.09$, $P = 0.17$). Average telomere length was not correlated with BMI in cases ($r = 0.124$, $P = 0.07$) but significantly correlated with BMI in controls ($r = 0.30$, $P < 0.001$). TLV was not correlated with BMI in either cases ($r = -0.02$, $P = 0.78$) or controls ($r = -0.06$, $P = 0.35$). There were no significant differences for the means of the average telomere length and TLV between male and female in cases or in controls, respectively.

Association between TLV and bladder cancer risk

Overall, TLV was significantly higher in cases (mean ± SD = 59.9 ± 5.01) than in controls (mean ± SD = 57.1 ± 3.98, $P < 0.001$). The case–control differences remained highly significant in each of the subgroups when stratified by age, gender and smoking status (Table 2).

Multivariate logistic regression analysis revealed that high TLV in blood lymphocytes was significantly associated with an elevated bladder cancer risk, with an adjusted odds ratio (OR) of 2.22 (95% CI: 1.48–3.35, Table 3) when comparing subjects who had high TLV (using the median value of all subjects as a cut point) with those who had low TLV, after adjustment for age, gender, smoking status, education and BMI. When subjects were categorized into quartiles of TLV, a significant trend of association between TLV and bladder cancer risk was present ($P_{trend} < 0.001$, Table 3), with adjusted OR = 4.21 (95% CI = 2.27–7.79) when comparing the highest quartile to the lowest quartile. The case-control comparisons of TLV by quartiles are shown in Supplementary Figure 1, available at *Carcinogenesis* Online. The risk association was stronger in younger subjects (age ≤ 60 years of age) than in older subjects (age > 60 years of age, Table 3). Alternatively, we used median or quartile values of control subjects as cutoff points when creating the comparison groups and obtained similar results.

Association between average telomere length and bladder cancer risk

Overall, average telomere length was significantly longer in cases (mean ± SD = 2210 ± 502) than in controls (1931 ± 643, $P < 0.001$). Similar case–control differences were observed for younger (age ≤ 60 years) and older (age > 60 years) subjects, males, former smokers and non-smokers (Table 2). No significant case–control difference in average telomere length was seen among females and current smokers.

Multivariate logistic regression analysis revealed that longer average telomere length was significantly associated with an elevated bladder cancer risk, with an adjusted odds ratio (OR) of 3.19 (95% CI: 2.07–4.91, Table 3), after adjustment for age, gender, smoking status, education and BMI. When subjects were categorized into quartiles of average telomere length, a significant trend of association between telomere length and bladder cancer risk was present ($P_{trend} < 0.001$, Table 3). The case–control comparisons of telomere length by quartiles are shown in Supplementary Figure 2, available at *Carcinogenesis* Online.

In addition to choosing the median or quartile values of all subjects as cutoff points to create comparison groups as in

Table 1. Demographic characteristics of study subjects

	Cases N = 224	Control N = 254	P
Age, mean (SD)	61.3 (10.4)	61.45 (10.7)	0.88
Age distribution, N (%)			
≤50	30 (13.4)	30 (11.8)	
51–60	76 (34.0)	92 (36.2)	
61–70	68 (30.4)	76 (29.9)	
71–80	44 (19.6)	52 (20.5)	
>80	6 (2.7)	4 (1.6)	0.89
Gender, N (%)			
Male	194 (86.6)	218 (85.8)	
Female	30 (13.4)	36 (14.2)	0.81
Cigarette use per day, N (%)			
1–10	54 (34.0)	27 (24.1)	
11–20	79 (49.7)	77 (68.8)	
21–39	17 (10.7)	7 (6.3)	
≥40	9 (5.7)	1 (0.9)	0.008
Tobacco use status, N (%)			
None user	49 (21.9)	117 (46.1)	
Water pipe only	16 (7.1)	25 (9.8)	
Cigarette only	124 (55.4)	92 (36.2)	
Both	35 (15.6)	20 (7.9)	<0.001
Education, N (%)			
None	140 (62.8)	115 (45.3)	
Literacy classes/primary school	64 (28.7)	83 (32.7)	
Preparatory/high/technical school	17 (7.6)	47 (18.5)	
College or university	2 (0.9)	9 (3.5)	<0.001
Marital status, N (%)			
Single/never married	1 (0.5)	1 (0.4)	
Married	198 (88.4)	226 (89)	
Widowed	24 (10.7)	26 (10.2)	
Divorced or separated	1(0.5)	1 (0.4)	0.997
BMI[a], mean (SD)	25.4 (4.0)	26.7 (5.1)	0.002
Muscle invasiveness, N (%)			
Non-invasive	53 (31.4)	—	
Invasive	116 (68.6)	—	—

[a]Body mass index.

Table 3, we used the median or quartile values of controls as cutoff points. Similar results were observed.

Joint effects of average telomere length and TLV on bladder cancer risk

To assess if combinations of telomere length and TLV increased the risk stratification for bladder cancer, we used the median value of all subjects as cutoff points to create comparison groups. Table 4 shows that long telomere length and high TLV jointly increased the risk of bladder cancer by more than 14-fold compared with individuals who had short telomere length and low TLV. In individuals who were 60 years of age or younger, those with longer telomere length and higher TLV had a 41-fold increased risk of bladder cancer compared with individuals who had short telomere length and low TLV. Among individuals who were older than 60 years of age, those with longer telomere length and higher TLV had 7-fold of increased risk of bladder cancer compared with individuals who had short telomere length and low TLV (Table 4). The details of the distribution of telomere length and TLV in cases and controls were illustrated in Supplementary Figure 3, available at *Carcinogenesis* Online.

Since the median value in controls has been frequently used as a cut point to create comparison groups in the literature, we also used this approach, resulting in a small number of cases (*N* = 5) having short telomere and low TLV. Thus the estimated ORs tended to be larger with wider 95% confidence interval. Nevertheless, similar magnitude and trend of risk association between joint effect of telomere length and TLV on bladder cancer risk were observed.

Discussion

The critical role of telomeres in aging and cancer has been established by studies using genetically engineered mouse models, human cell lines and tumor tissues. In contrast, the process of translating this knowledge to enhance the management of cancer and aging-associated disease has been slow and replete with challenges. Despite intense interest in telomeres and cancer, almost all the human studies that were designed to determine the relationship between telomere function and cancer risk have focused on measuring average telomere length across all the chromosomal ends. This approach has not been able to clearly establish whether average TL in blood leucocytes is a useful biomarker for the assessment of cancer risk and progression. Our study is the first to evaluate the TLV across all chromosomal ends as a bladder cancer risk biomarker. The results support our hypothesis that high TLV is significantly associated with an increased risk of bladder cancer and is consistent with our recent finding that high TLV was significantly associated with an increased risk of early onset lung cancer (29).

Table 2. Case–control comparison of telomere features, stratified by age, gender and smoking status

Telomere features	Cases mean (SD)	Controls mean (SD)	*P*
All subjects, *N*	224	253	
Avg_TL[a]	2210 (502)	1931 (643)	<0.001
TLV[b]	59.9 (5.0)	57.1 (4.0)	<0.001
Age ≤ 60, *N*	113	128	
Avg_TL	2305 (504)	1966 (699)	<0.001
TLV	59.28 (4.5)	56.66 (4.0)	<0.001
Age > 60, *N*	111	125	
Avg_TL	2113 (483)	1896 (581)	0.002
TLV	60.55 (5.4)	57.63 (3.9)	<0.001
Male, *N*	194	218	
Avg_TL	2197 (480)	1903 (618)	<0.001
TLV	59.88 (5.0)	57.12 (3.9)	<0.001
Female, *N*	30	35	
Avg_TL	2290 (631)	2109 (770)	0.307
TLV	60.13 (5.1)	57.25 (4.2)	0.016
Never smokers, *N*	65	141	
Avg_TL	2213 (555)	1953 (684)	0.008
TLV	59.46 (5.3)	56.82 (3.8)	<0.001
Former smokers, *N*	61	66	
Avg_TL	2150 (560)	1780 (489)	<0.001
TLV	60.45 (5.0)	58.24 (4.4)	0.009
Current smokers, *N*	98	46	
Avg_TL	2245 (423)	2081 (677)	0.136
TLV	59.87 (4.9)	56.54 (3.7)	<0.001

[a]Average telomere length; [b]telomere length variation.

High TLV is considered one of the characteristics of telomere dysfunction in cancer cells using alternative lengthening of telomeres mechanism to maintain telomere length (22,23). Using *in vitro* cell culture system, we consistently observed that significant increase in TLV occurred before significant loss of telomere length during *in vitro* cellular aging in normal human fibroblast cells (data not shown). Our published data demonstrated that over-expressing hTERT in normal human cells prominently and consistently decreased TLV, while the increase in average telomere length is not as consistent (21).

High variation in telomere length may represent a deficiency in telomere maintenance. This notion is supported by the observation that blood leucocytes of Dyskeratosis congenital patients exhibit sharp differences in telomere lengths (30). Dyskeratosis congenital is a rare genetic disorder characterized by cancer predisposition and accelerated aging due to defect in telomere maintenance. Previous studies have shown that the shortest telomeres, not average telomere length, drove chromosome instability in cancer cells (18–20) and only a small subset of dysfunctional telomeres can trigger telomere-associated DNA damage response, driving chromosomal instability (31–33). TLV, defined as coefficient of variation of all measured telomeres, measures the overall variability of telomeric DNA distribution across all chromosome ends. To select an appropriate parameter to access the TLV, we initially considered standard deviation, variance and coefficient of variation. Both standard deviation and variance are highly correlated with average telomere length, thus are not suitable as an independent telomere parameter. The value of TLV is driven by extreme values, such as very short

Table 3. Risk estimation of association between telomere features and bladder cancer, for all subjects and stratified by age, using the median or quartile values of all subjects as cutoffs

	Telomere length variation					Average telomere length				
	N					*N*				
All subject	Case	Control	OR	(95% CI)	*P*	Case	Control	OR	(95% CI)	*P*
By median										
<Median	138	100	Ref			142	96	ref		
≥Median	86	153	2.22	(1.48–3.35)	<0.001	82	157	3.19	(2.07–4.91)	<0.001
By quartile										
Q1	47	73	Ref			55	65	Ref		
Q2	57	62	1.60	(0.88–2.91)		74	44	3.55	(1.89–6.66)	
Q3	81	38	2.00	(1.11–3.62)		68	52	5.51	(2.93–10.38)	
Q4	39	80	4.21	(2.27–7.79)	<0.001[a]	27	92	7.50	(3.83–14.67)	<0.001[a]
Age ≤ 60										
By median										
<Median	63	40	Ref			78	47	Ref		
≥Median	43	81	3.09	(1.62–5.88)	<0.001	28	74	4.99	(2.53–9.85)	<0.001
By quartile										
Q1	22	34	Ref			17	32	Ref		
Q2	29	26	2.17	(0.89–5.31)		33	18	2.55	(0.93–6.98)	
Q3	34	14	3.74	(1.51–9.22)		45	29	6.08	(2.23–16.53)	
Q4	21	47	5.79	(2.22–15.08)	0.002[a]	11	42	10.26	(3.81–27.63)	<0.001[a]
Age > 60										
By median										
<Median	75	60	Ref			64	49	Ref		
≥Median	43	72	1.91	(1.09–3.36)	0.024	54	83	2.23	(1.25–3.96)	0.006
By quartile										
Q1	25	39	Ref			38	33	Ref		
Q2	28	36	1.34	(0.38–1.78)		41	26	4.68	(2.04–10.71)	
Q3	47	24	1.49	(0.64–3.47)		23	23	5.31	(2.29–12.31)	
Q4	18	33	3.26	(1.42–7.51)	0.025[a]	16	50	5.30	(2.03–13.87)	<0.001[a]

Odds ratios (ORs) were adjusted for age, gender, BMI, smoking status and education. The median or quartile values of all subjects were used as cutoffs when creating groups.
[a]P-for-trend.

Table 4. Joint effects of average telomere length and telomere length variation on bladder cancer risk, using the median of all subjects as cutoffs

	N				
Avg_TL/TLV	Case	Control	OR	(95% CI)	P-for-trend
All subject					
Short/low	16	80	Ref		
Short/high	66	77	3.55	(1.79–7.04)	
Long/low	70	73	4.77	(2.37–9.59)	
Long/high	72	23	14.68	(6.74–31.98)	<0.001
Age ≤ 60					
Short/low	4	43	Ref		
Short/high	24	31	5.99	(1.83–19.60)	
Long/low	39	38	8.38	(2.68–26.19)	
Long/high	39	9	41.08	(10.68–158.07)	<0.001
Age > 60					
Short/low	31	35	ref		
Short/high	33	14	2.74	(1.14–6.58)	
Long/low	12	37	3.12	(1.23–7.87)	
Long/high	42	46	7.52	(2.74–20.64)	<0.001

The penalized likelihood approach was used in the estimates when data are sparse (cell count <5). The median values of all subjects were used as cutoffs when creating groups. ORs were adjusted for age, gender, BMI, smoking status and education.
Avg_TL, average telomere length; TLV, telomere length variation.

or extremely long telomeres. We found that TLV is highly correlated with the frequency of very short telomeres (Spearman corr $r = 0.85$, data not shown) and individuals who had high TLV also had high numbers of chromosomal ends possessing very short, probably dysfunctional telomeres, even when the average telomere length seemed reasonable (within normal range).

We also found that TLV is highly correlated with frequency of excessively long telomeres ($r = 0.77$, data not shown). While the detrimental effects of short telomeres on human health have been intensively studied, the health consequences of excessively long telomeres remain to be illustrated. Excessively long telomeres in ALT cells suffer a decreased saturation of shelterin proteins, leading to reduced compaction of telomeric chromatin and increased telomere fragility as suggested by previous studies (34,35). This shelterin protein unsaturation or 'intermediate status' of telomeres resulted in impaired chromosome end protections that are susceptible to DNA damage response, leading to genomic instability (36). Recent data by our group demonstrated that telomerase, a key telomere maintenance enzyme, not only elongates short telomeres, but also shortens excessively long telomeres in human cells, indicating maintaining telomere homeostasis is critical for a normal cellular function (21). TLV measures the combined effects of very short and excessively long telomeres and represents a novel aspect of telomere function and might be a useful biomarker for cancer risk. It should be noted that our study used case-control study design and is cross-sectional in nature. Thus it is not possible to establish whether TLV is a causative factor for bladder cancer. Nevertheless, the promising results warrant future investigation, preferably with a cohort study design.

We examined relationship between average telomere length in blood lymphocytes and bladder cancer risk in this Egypt population and found the long average telomere length is significantly associated with an increased risk of bladder cancer. This finding is not consistent with previous studies which reported that the shorter average telomere length in blood leucocytes was significantly associated with an increased bladder cancer risk (14–16). These previous studies were based on United States (15,16) or Swedish (14) populations. The positive association observed in our study is mainly due to the noticeably short average telomere length in the control subjects compared with that in controls of US population of comparable age (data not shown). Positive associations between average telomere length in blood leucocytes and cancer risk has been reported for breast (37), lung (38), soft tissue sarcoma (39) and pancreatic (40) cancers. No reported studies examined association between average telomere length in blood leucocytes and bladder cancer risk in Egypt population. It should be noted that in the present study, cultured T lymphocytes were used for the measurement of telomere length by T-FISH, while previous studies used DNA purified from blood leucocytes for the measurement of telomere length by PCR. It has been reported that telomere length differs significantly between different subtypes of blood leucocytes, with telomere length in B lymphocytes > T lymphocytes > neutrophils (41–43). In addition, lymphocytes having long telomeres may survive better and were selected for by the blood culture process. These two factors are the main differences of the present study compared with previous reported bladder cancer studies and may contribute to the disparate findings. Due to the moderate sample size, the results need to be interpreted with caution.

Importantly, we found combination of TLV and average telomere length strikingly enhances risk stratification for bladder cancer. Long and heterogeneous telomere length in blood lymphocytes was strongly associated with an increased risk of bladder cancer (Table 4). It is worth noting that long and heterogeneous telomeres are commonly observed in telomerase negative and ALT+ cancer cells. Our study provides some new insight regarding the assessment of telomere function in blood lymphocytes that is relevant to cancer. If confirmed by future studies, TLV in combination with average telomere length in blood lymphocytes may become promising biomarkers for bladder cancer risk assessment.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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