*Short Communication*

# Differential Induction in Telomerase Activity among Bladder Cancer Patients and Controls on γ-Radiation

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## Abstract

**Expression of telomerase is one of the hallmarks of tumor cells and has been used as a diagnostic biomarker and a therapeutic target in cancer. Novel findings have shown that telomerase activation in normal human epithelial cells may affect expression of several cancer-related genes, such as growth-related genes and c-*myc* gene, suggesting a possible role of telomerase in tumor initiation. Therefore, we hypothesized that individuals who are sensitive to mutagen challenge in terms of induced telomerase activity might have increased cancer risk. We tested this hypothesis in a bladder cancer case-control study (51 cases and 51 matched controls) by measuring baseline and γ-radiation–induced telomerase activities in peripheral blood lymphocytes. We found a significantly higher γ-radiation–induced telomerase activity in bladder cancer cases compared with the controls (1.34 versus 1.23; $P = 0.044$). A similar finding was also observed using the normalized telomerase activity (ratio of γ-radiation induced versus baseline; 1.49 versus 1.19; $P < 0.001$). In further categorizing the telomerase activity using 75% of the normalized value in the controls as a cutoff point, we found a significantly increased risk for bladder cancer associated with higher induced telomerase activity (adjusted odds ratio, 3.62; 95% confidence interval, 1.38-9.51). In quartile analysis, a dose-response association was noted between the induced telomerase activity and increased bladder cancer risk ($P_{trend} = 0.005$). Our findings provide the first evidence linking the mutagen-induced telomerase activity in peripheral blood lymphocytes to the risk of bladder cancer, which warrants further investigation in large-sized studies and other cancer types.** (Cancer Epidemiol Biomarkers Prev 2007;16(3):606–9)

## Introduction

Telomerase is a ribonucleoprotein composed of a reverse transcriptase (TERT) and a RNA template. Activation of telomerase is a critical event in human cell immortalization and carcinogenesis (1). Telomerase is normally not expressed in normal human somatic cells; however, telomerase activity has been detected in ~85% of patients suffering from various cancers, including breast, prostate, lung, liver, pancreatic, bladder, and colon cancer. The increased telomerase activity in cancer cells not only helps to maintain the telomere at a constant length over indefinite cell divisions but also plays a role in the multistep development of tumors (2).

For example, introduction of *hTERT* gene into human mammary epithelial cells has been shown to cause overexpression of the oncogene c-*myc* (3). It has also been reported that telomerase may promote cell proliferation by modulating the expression of growth-controlling genes, such as *EGFR, FGF*, and *IL-1Ra*, in human normal cells (4). In addition, telomerase expression has been linked to chromosome healing and increased cell survival (5, 6). Cell cycle analysis has further associated high telomerase activity with an increased rate of cells entering the S phase and a high degree of malignancy (7). These findings suggest that telomerase may affect both cancer-related genes and biological pathways in addition to maintaining telomere length and might consequently contribute to the initiation of tumor development.

More importantly, telomerase expression could be induced by environmental mutagens, such as irradiation and *N*-nitrosobis (2-oxopropyl) aminecan, which are also carcinogens (8). Therefore, we hypothesized that individuals with higher mutagen-induced telomerase activity might have an increased risk of cancer development.

Although the role of telomerase in tumorigenesis has been extensively investigated using tumor tissues and immortalized cell lines, no molecular epidemiologic study has been done to determine whether inherited discrepancy on mutagen-induced telomerase activity in normal cells is associated with cancer risk. In this study, we tested this hypothesis in our ongoing bladder cancer case-control study by measuring baseline and γ-radiation–induced telomerase activity in peripheral blood lymphocytes.

## Materials and Methods

**Study Population.** The bladder cancer cases were recruited from The University of Texas M. D. Anderson Cancer Center and Baylor College of Medicine. All cases were newly diagnosed and histologically confirmed bladder cancer patients who had not received chemotherapy or radiotherapy before enrollment. There were no age, gender, ethnicity, or cancer stage restrictions. The controls are recruited in collaboration with the Kelsey-Seybold clinics, the largest private multispecialty physician group in the Houston metropolitan area. The majority of controls visit the clinics for annual health check-ups. Controls had no previous cancer history (except nonmelanoma skin cancer) and were frequency

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**Table 1. Distribution of select characteristics among study subjects**

| Variables | Bladder cancer | | |
|---|---|---|---|
| | Cases ($n$ = 51) | Controls ($n$ = 51) | $P$* |
| Sex, $n$ (%) | | | |
| Male | 36 (70.59) | 36 (70.59) | |
| Female | 15 (29.41) | 15 (29.41) | 1.000 |
| Age, y | | | |
| Mean (SD) | 63.98 (10.41) | 64.10 (10.41) | 0.907 |
| Smoking status, $n$ (%) | | | |
| Never | 13 (25.49) | 24 (47.06) | |
| Ever | 38 (74.51) | 27 (52.94) | 0.023 |
| Pack-years † | | | |
| Mean (SD) | 37.92 (25.96) | 24.01 (21.23) | 0.024 |

*$P$ values were derived from the Pearson $\chi^2$ test for categorical variables (sex and smoking status) and Student's $t$ test for continuous variables (age and cigarette smoking pack-years). All $P$ values are two sided.
† In ever smokers only.

matched to cases by age (±5 year), gender, and ethnicity. For the parent study, to date, there are 1,067 cases and 869 controls enrolled. The response rates for the cases and controls have been 92% and 75%, respectively. Blood samples were obtained for 91.0% cases and 99.5% controls. In the present pilot study, 51 case-control pairs were randomly chosen from the existing pool of study participants consecutively recruited from 1999 to 2004. All study subjects included in the current analysis were Caucasians and controls were individually matched to bladder cancer cases on age (±1 year) and gender. All participants were interviewed, and information about demographics, smoking history, and occupational exposures were collected. The study was approved by all relevant review boards, and the signed informed consent was obtained from each participant.

**Blood Collection and Lymphocyte Isolation.** Ten milliliters of blood were collected in a sodium-heparinized tube from each study subject. Researchers doing the laboratory assays were blinded to the case-control status of the coded blood samples. Lymphocytes were isolated using the Ficoll-Hypaque standard technique. The isolated lymphocytes were then stored in liquid nitrogen with $4 \times 10^6$ cells in each vial.

**Cell Culture.** The frozen lymphocytes were thawed and then incubated for 72 h at 37°C in RPMI 1640 supplemented with 20% fetal bovine serum and 112.5 μg/mL phytohemagglutinin (Murex Diagnostics, Norcross, GA). For each lymphocyte sample, two flasks were cultured using $2 \times 10^6$ cells submerged in 4 mL medium. Cultured lymphocytes were irradiated with γ-radiation from a $^{137}$Cs source (Cesium Irradiator Mark 1, model 30; J.L. Shepherd and Associates, Glendale, CA). The optimal dose and time point were 0.5 Gy and 12 h after exposure to γ-radiation, respectively. Cell cultures were directly exposed to incident radiation for 14 s at a rate of 0.0357 Gy/s and then allowed to grow for an extra 12 h before being harvested. Unirradiated cells were also harvested at the same time point using conventional procedures. The cell pellets were resuspended in 200 μL lysis buffer and incubated for 30 min on ice. After centrifugation at 16,000 × $g$ for 20 min at 4°C, the supernatants of lysates were collected. The protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

**Telomerase Activity Measurement.** Telomerase activity was assessed using the telomerase PCR-ELISA kit from (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. In brief, the protein concentration was first adjusted to the same level for each sample. The extracted proteins were then incubated with biotinylated telomerase substrate oligonucleotide (P1-TS primer) at 25°C for 20 min. The extended products were amplified by PCR using P1-TS and P2 primers for 30 cycles under the following conditions: 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. Biotinylated telomeric repeat amplification products were incubated for 2 h at 37°C with a digoxigenin-labeled detection probe complementary to the telomeric repeat sequence and immobilized onto streptavidin-coated microtiter plates. Next, the wells were incubated for 30 min at room temperature with peroxidase-labeled anti-digoxigenin polyclonal antibody. Finally, the amount of telomeric repeat amplification products was determined after the addition of the peroxidase substrate (3,3′,5,5′-tetramethylbenzidine). The absorbance of each batch was measured at a wavelength of 450 nm (reference wavelength, 595 nm; Vmax Kinetic microplate reader, Molecular Devices, Sunnyvale, CA). For each sample, a heat-treated (85°C for 10 min) negative control was included. Additionally, samples of paired cases and controls were tested in the sample batch throughout the study. Relative telomerase activity within different samples was defined in the following way: (absorbance of sample − absorbance of heat-treated sample) / absorbance of internal standard of the sample. To calculate the intra-assay variation, five samples with relatively low, medium, and high telomerase activities were chosen and telomerase activity was measured. The intra-assay coefficient of variation was determined through three replicated analyses of samples during the same day.

**Statistical Analysis.** All statistical analyses were done using the Stata 8.0 statistical software package (Stata Corp., College Station, TX). The Pearson $\chi^2$ test was used to test for differences in distribution between the cancer patients and the control subjects with regard to gender and smoking status. The Student's $t$ test was used for testing differences in distribution between cases and controls with regard to age and pack-years (among ever smokers). The Wilcoxon matched-pair signed-rank test was used to compare telomerase activities and its ratio of baseline and treatment samples and analyze the correlation between telomerase activity and gender or smoking status. The ratio of telomerase activity was also analyzed as a categorical variable. The association between cancer risk and induced telomerase activity was estimated by adjusted ORs along with the corresponding 95% confidence intervals. To remove the confounding effects of other factors, conditional logistic regression analysis with multiple covariates was done. Spearman's correlation test was used to examine the correlation between telomerase activity and age. Pack-years and smoking status were defined as described previously (9). All statistical tests were two sided.

**Table 2. Telomerase activity in cases and controls for bladder cancer studies**

| Telomerase activity | Cases | | | Controls | | | $P$* |
|---|---|---|---|---|---|---|---|
| | $n$ | Median (range) | $P$ † | $n$ | Median (range) | $P$ † | |
| Baseline | 51 | 0.94 (0.21-3.16) | | 51 | 1.06 (0.14-4.69) | | 0.358 |
| After γ-radiation treatment | 51 | 1.34 (0.35-4.86) | <0.001 | 51 | 1.23 (0.09-6.89) | <0.001 | 0.044 |
| Ratio (γ-radiation/baseline) | 51 | 1.49 (0.74-8.56) | | 51 | 1.19 (0.35-2.05) | | <0.001 |

*$P$ values were derived from the Wilcoxon matched-pair signed-rank test.
†$P$ values were calculated for assessing the difference of telomerase activity between baseline and after γ-radiation treatment in cases or controls.

**Table 3. Relative bladder cancer risk estimates for ratio of telomerase activity**

| Ratio of telomerase activity (γ-radiation/baseline) | Cases *n* (%) | Controls *n* (%) | OR (95% CI)* |
|---|---|---|---|
| By 75th percentile value | | | |
| Low | 24 (47.06) | 39 (76.47) | 1 (Reference) |
| High | 27 (52.94) | 12 (23.53) | 3.62 (1.38-9.51) |
| By quartiles | | | |
| 1st + 2nd Quartiles | 11 (21.57) | 26 (50.98) | 1 (Reference) |
| 3rd Quartile | 13 (25.49) | 13 (25.49) | 2.04 (0.68-6.11) |
| 4th Quartile | 27 (52.94) | 12 (23.53) | 5.25 (1.64-16.79) |
| $P_{trend}$ | | | $P = 0.005$ |

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
*Adjusted by age, gender, and smoking status.

## Results

The pilot study included 51 Caucasian bladder cancer cases and 51 Caucasian controls (Table 1). Except that the percentage of females is slightly higher than that of the parent study (29% versus 22%), the mean age and the distribution of smoking variables in cases and controls did not differ from the parent study population. By study design, the cases and controls included in current analysis were well matched on gender ($P$ = 1.00) and age ($P$ = 0.907). However, there were statistically significant differences between the cases and the controls in terms of smoking status and pack-years. Ever smokers were significantly overrepresented among bladder cancer cases (74.51%) compared with control subjects (52.94%; $P$ = 0.023). Bladder cancer patients were also self-reported heavier smokers than control subjects (pack-years: 37.92 ± 25.96 versus 24.01 ± 21.23; $P$ = 0.024).

The baseline and γ-radiation–induced telomerase activities in peripheral blood lymphocytes were measured by the telomerase PCR-ELISA assay (Table 2). For this assay, the mean intra-assay coefficient of variation is 12.7%, similar to those reported in previous studies (10, 11). The negative controls of all samples are all testing negative, with <0.1 absorbance units. These results suggest the high reliability of our assay. In the present study, we found that the baseline telomerase activity of peripheral blood lymphocytes in bladder cancer cases was not significantly different from that in controls (0.94 versus 1.06; $P$ = 0.358). On γ-radiation, telomerase activity was significantly increased among bladder cancer cases (1.34 versus 0.94; $P < 0.001$) and controls (1.23 versus 1.06; $P < 0.001$) compared with the baseline level. However, the ratio of telomerase activity (γ-radiation induced/baseline) was significantly higher in the bladder cancer cases than in the controls (1.49 versus 1.19; $P < 0.001$).

When the ratio of telomerase activity was analyzed as a categorical variable using the 75th percentile of the value in the controls as the cutoff, a high level of induced telomerase activity was observed among 52.94% of the bladder cancer cases compared with 23.53% of the controls (Table 3). This high level of induced telomerase activity was significantly associated with an increase risk for bladder cancer (odds ratio, 3.62; 95% confidence interval, 1.38-9.51) in a conditional logistic regression analysis with adjustment for confounding factors of age, sex, and smoking status. When we further categorized subjects into quartiles of the ratio of telomerase activity, based on its distribution in the controls (with the combined first and second quartiles as the reference category), we detected a dose-response relationship between the level of induced telomerase activity and bladder cancer risk. The adjusted odds ratio (95% confidence intervals) for individuals in the third and fourth quartiles of the ratio of telomerase activity was 2.04 (0.68-6.11) and 5.25 (1.64-16.79), respectively ($P_{trend}$ = 0.005; Table 3).

The baseline and γ-induced telomerase activities were also assessed by age, gender, and smoking status. There were no significant associations between telomerase activity and age, gender, or smoking status (data not shown).

## Discussion

Our results are consistent with previous observations that telomerase activity was γ-radiation inducible in hematopoietic cells (12). The up-regulation of telomerase activity has also been observed in human lymphoblasts (13) and tumor cell lines (14) after X-ray irradiation. However, the underlying mechanisms of radiation-induced telomerase activity are still unknown. It has been suggested that both transcriptional activation and posttranslational control of *TERT* might be involved in this process (15).

The difference of γ-radiation–induced telomerase activities between bladder cancer cases and controls could be associated with the expression of tumor suppressor gene *p53*. Our previous study showed that the p53 expression was induced by γ-radiation in lymphoblastoid cell lines derived from both cancer patients and healthy controls (16). The level of p53 up-regulation was significantly less in cell lines from cases than those from controls. It was also reported recently that the overexpression of wild-type *p53* could down-regulate the telomerase activity in various cancer cell lines through transcriptional repression of *hTERT* (17). These findings could account in part for the low level of γ-radiation–induced telomerase activity in bladder cancer controls detected in our current study because controls may have overexpressed *p53* on mutagen exposure. This p53-telomerase connection could provide a possible mechanism for our present findings.

Our previous study has shown that telomere shortening is associated with increased risks for several cancer types, including bladder cancer (18). Analysis from our current study showed that telomere length and γ-radiation–induced telomerase activity were significant independent predictors of bladder cancer risk when we included both variables in the same model (data not shown). This result further supports that telomerase may promote bladder cancer development in a way independent of telomere shortening. Findings from our molecular epidemiologic study are also congruent with previous observations in cell line and animal studies. A previous study found that telomerase could facilitate the telomere length-independent tumorigenicity of Ras-transformed, immortalized GM847 cells using an *in vitro* tumorigenesis assay (19). An *in vivo* experiment also showed that *p53* mutant transgenic mice expressing mTERT had high tumor incidence on exposure to chemical carcinogenesis, which was also a tumorigenic process independent of telomere elongation (20). Moreover, it was reported that high levels of telomerase expression in mouse T cells may lead to spontaneous T-cell lymphoma, without telomere lengthening (21). All these evidences support the hypothesis that expression of telomerase may affect cancer-related genes and biological pathways and consequently initiate and promote tumorigenesis.

Because smoking status was significantly different between bladder cancer cases and controls, we stratified our data by smoking status to ensure that smoking behavior did not contribute to the differences in telomerase activity between the case patients and the control subjects. There were no

significant differences in baseline and γ-radiation–induced telomerase activity between ever smokers and never smokers (data not shown). Thus, smoking status apparently does not explain the substantial differences in telomerase activity between bladder cancer cases and controls.

In conclusion, γ-radiation–induced telomerase activity in peripheral blood lymphocytes is shown for the first time to be associated with risk of bladder cancer. This finding suggests that the induced telomerase activity measured by the telomeric repeat amplification protocol–ELISA assay might be a promising phenotypic biomarker for determining an individual's susceptibility to bladder cancer. Further investigations are warranted to confirm our results in large case-control studies and other cancer types.

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