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Telomere length and recurrence risk after curative resection in patients with early-stage non-small cell lung cancer: a prospective cohort study

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Abstract

Background—We hypothesized that telomere length in peripheral blood would have significant predictive value for risk of recurrence after curative resection in non-small cell lung cancer (NSCLC).

Methods—This prospective study included 473 patients with histologically confirmed early stage NSCLC who underwent curative resection at MD Anderson Cancer Center between 1995 and 2008. Relative telomere length (RTL) of peripheral leukocytes was measured by real-time PCR. The risk of recurrence was estimated as hazard ratios (HRs) and 95% confidence intervals (CIs) using a multivariable Cox proportional hazard regression model.

Results—Median duration of follow-up was 61 months and 151 patients (32%) had developed recurrence at time of analysis. Patients who developed recurrence had significantly longer mean RTL compared to those without recurrence (1.13 vs 1.07, P=0.046). A subgroup analysis indicates that women had longer RTL compared to males (1.12 vs 1.06, P=0.025), and the patients with adenocarcinoma demonstrated longer RTL compared to those with other histologic types (1.11 vs 1.05, P=0.042). To determine if longer RTL in women and adenocarcinoma subgroup would predict risk of recurrence, multivariate Cox analysis adjusting for age, gender, stage, pack year and treatment regimens was performed. Longer telomeres were significantly associated with

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higher risk of developing recurrence in female (HR=2.25; 95% CI, 1.02-4.96, *P*=0.044) and adenocarcinoma subgroups (HR=2.19; 95% CI, 1.05-4.55, *P*=0.036). The increased risk of recurrence due to long RTL was more apparent in females with adenocarcinoma (HR=2.67; 95% CI, 1.19-6.03, *P*=0.018).

Conclusions—This is the first prospective study to suggest that long RTL is associated with recurrence in early stage NSCLC after curative resection. Women and adenocarcinoma appear to be special subgroups in which telomere biology may play an important role.

Introduction

Lung cancer is the leading cause of cancer-related death in the United States, with non-small cell lung cancer (NSCLC) accounting for 87% of all cases.[1] Surgical resection in early stage offers the best chance of cure in NSCLC. However, even with complete resection, patients are at significant risk of recurrence and death from NSCLC.[2] Adjuvant cisplatin-based chemotherapy for completely resected early stage NSCLC can offer survival benefit,[3-5] but it is not clear which subgroup of patients would be at increased risk of developing recurrence. Hence, it is important to identify a high-risk group of patients who would most likely develop recurrence and potentially benefit from adjuvant chemotherapy. Likewise, a low-risk subgroup may be spared of toxic chemotherapy regimen. A biomarker-driven risk prediction model is necessary in molecularly-heterogeneous NSCLC.

Telomere pathway is implicated in pathogenesis of various types of human cancer. Telomeres are specialized structures located at chromosome ends consisting of nucleotide repeats (TTAGGG)n and ordered protein complex that help maintain genomic structural integrity by protecting chromosome ends from degradation.[6] Progressive shortening of telomeres results in critically short telomeres leading to cellular crisis.[7] However, in cancer cells, up-regulation of telomerase is found in approximately 90% of human cancers leading to continued cellular proliferation.[8,9] Several studies have shown an association between shorter telomeres in peripheral leukocytes and risk of various types of cancer including lung cancer.[10,11]

There are a number of telomere-associated proteins that have essential roles in maintaining telomere length. For example, TRF1 and TRF2 are thought to be suppressors of telomere elongation.[12,13] POT1 binds the 3’ telomeric overhang and may regulate telomerase access.[14,15] TPP1 is identified as a regulator of POT1.[16] Human RAP1 is recruited to the telomere by TRF2 without directly binding to DNA[17] and targeting human RAP1 with small interference RNA leads to longer telomeres.[18] Such complexity suggests a highly-regulated mechanism of telomere pathway that may impact clinical outcome.

There exists a significant interindividual variation in telomere length.[19,20] Furthermore, genome-wide association studies (GWAS) identified single nucleotide polymorphisms (SNPs) in genes encoding telomere-associated proteins that are implicated in maintaining leukocyte telomere length,[21,22] and risk of pancreatic,[23] skin[24] and lung cancer.[25] We hypothesize that telomere length in peripheral leukocytes would have significant predictive value for recurrence following curative surgical resection in NSCLC, thereby guiding

decision for adjuvant chemotherapy. To our knowledge, this is the first investigation of the association between telomere length and clinical outcome in early stage NSCLC.

Materials and methods

Study population

This prospective study included 467 patients with histologically confirmed NSCLC who were enrolled from 1995 to 2008 in an ongoing epidemiologic lung cancer study at MD Anderson Cancer Center. Written informed consent was signed by all study participants and the study was reviewed and approved by the Institutional Review Board of MD Anderson Cancer Center.

All the study participants had early stage disease (stage I and II) and underwent surgery or surgery plus chemotherapy (mostly platinum-based). Each patient was interviewed by trained MD Anderson staff interviewers to collect comprehensive epidemiologic data using structured questionnaire. Follow-up data was abstracted from medical records for each patient. After the in-person interview, 40 ml blood sample was prospectively collected from each participant and sent to the laboratory for molecular analysis.

Telomere length assessment

High-quality genomic DNA was extracted from peripheral blood using the QIAamp Maxi DNA kit (Qiagen) according to the manufacturer's protocol. The details of telomere length assessment have been described previously.[26] Briefly, the relative overall telomere length was assessed using a modified version of the real-time quantitative PCR and was determined by the normalized ratio between the telomere repeat copy number and the single gene (human globulin) copy number to standardize between different runs. The PCR reaction mixture (15 μL) for the telomere amplification consisted of 5 ng of genomic DNA, 1×SYBR Green Mastermix (Applied Biosystems), 200 nmol/L Tel-1, and 200 nmol/L Tel-2. The PCR reaction mixture for human globulin amplification consisted of 5 ng of genomic DNA, 1×SYBR Green Mastermix, 200 nmol/L Hgb-1, and 200 nmol/L Hgb-2. 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 PCRs for telomere and Hgb were done on separate 384-well plates, with the same samples in the same well positions. Negative controls, positive controls, a calibrator DNA, and a standard curve were included in each run. A six-point standard curve was created by diluting the reference DNA sample (the same DNA sample for all runs) using a 2-fold increment per dilution (from 20 to 0.625 ng) in each reaction.

Statistical Analysis

The primary endpoint was recurrence (time from diagnosis to recurrence or last follow-up). Recurrence was defined as evidence of local recurrence or new sites of involvement in lymph nodes or distant organ after curative resection. Patients who were lost to follow-up or who were alive at the end of the study without evidence of recurrence were censored. The association between telomere length and age was assessed by linear regression. Student's *t* test was used to assess the association of telomere length with recurrence. The multivariate

Cox proportional hazard model while adjusting for age, gender, ethnicity, stage, pack year, and treatment regimens was used to assess the effect of telomere length on recurrence. Patients with previously diagnosed stage I and II disease who came to MD Anderson for treatment after developing recurrence were excluded from the Cox proportional hazard analysis of recurrence. All the statistical analyses above were performed using STATA software (version 10.1, Stata Corporation, College Station, TX).

Results

Patient characteristics

The characteristics of the subjects are shown in Table 1. A total of 473 patients who were enrolled between 1995 and 2008 in an ongoing prospective cohort study and underwent curative therapy for early stage NSCLC at MD Anderson Cancer Center were included. Adenocarcinoma was the most common histology involving 61.5% of patients. A majority of patients (73.3%) underwent surgical resection only and the remaining 26.7% of patients received either neoadjuvant or adjuvant chemotherapy. Median follow-up time was 61 months.

Association of telomere length with recurrence and other clinicopathologic parameters

RTL results from a total of 473 peripheral blood samples that were collected at time of in-person interview were used for the analysis. Age and RTL showed inverse association (theta= - 0.00807774; *P*=1.87E-08). At time of analysis 151 patients (32%) developed recurrence. Our results show that recurrence group demonstrated significantly longer mean RTL compared to non-recurrence group (1.13 vs 1.07, *P*=0.0465) (Table 2 and Figure 1). In addition, females had longer RTL compared to males, and the patients with adenocarcinoma demonstrated longer RTL compared to those with other histologic types (Table 2 and Figure 1). There was no significant association of RTL with smoking status, ethnicity, stage of disease and type of treatment (surgery alone vs surgery plus chemotherapy) (Table 2). After adjusting for age, the recurrence remained in borderline association (*P*=0.076) with telomere length while the correlation with sex (*P*=0.11) and histology (*P*=0.11) no longer became significant.

Effect of telomere length on risk of recurrence

The effect of RTL on risk of recurrence after surgical resection in NSCLC was assessed. Multivariable Cox proportional analysis on 427 patients after adjusting for age, gender, ethnicity, stage, pack year, and treatment regimens showed that longer telomeres were associated with higher risk of developing recurrence at borderline statistical significance (HR=1.75; 95% CI, 0.96-3.22, P=0.070) (Table 3). Given the results from the previous section demonstrating association of long telomeres with female gender and adenocarcinoma histology, a subgroup analysis was conducted. The increased risk of recurrence due to long RTL was more pronounced and statistically significant in female (HR=2.25; 95% CI, 1.02-4.96, *P*=0.044) and adenocarcinoma (HR=2.19; 95% CI, 1.05-4.55, *P*=0.036) subgroups (Table 3). Moreover, in females with adenocarcinoma, a higher risk of recurrence due to long RTL was estimated and was highly significant (HR=2.67; 95% CI, 1.19-6.03, P=0.018) (Table 3). Kaplan-Meier curves and log-rank tests

comparing long versus short RTL dichotomized by the median telomere length are shown in Figure 2. Figure 2D reinforces the finding that the difference in the risk of recurrence between long versus short RTL is particularly significant in female patients with adenocarcinoma histology (P=0.033). Long RTL was borderline associated with recurrence in an ever-smoker subgroup (P=0.054).

Discussion

We report novel findings that early stage NSCLC patients who develop recurrence after curative therapy had longer blood telomeres compared to the patients without recurrence. Although several past studies associated short telomeres with increased risk of lung cancer,[10,27,28] we need to emphasize that our study subjects had newly diagnosed early stage disease, and the blood samples were collected before undergoing chemotherapy excluding the possibility of chemotherapy-induced change in telomere length. In addition, Pooley and colleagues reported that mean telomere length was shorter in retrospectively collected cases than in controls, but the association was markedly weaker in the prospective studies, suggesting telomere shortening may occur after diagnosis.[29] A recent prospective cohort study involving Chinese females demonstrated that longer RTL in blood may be associated with increased risk of lung cancer.[30] In addition, two other studies by Svenson and colleagues[31,32] showed that longer RTL was associated with worse prognosis in breast cancer and clear cell renal cell carcinoma (ccRCC) consistent with our finding. Interestingly, the study involving ccRCC showed that long blood RTL was associated with poor survival only in patients with non-metastatic ccRCC, possibly suggesting dynamic changes in telomere biology during tumor progression. Though telomere biology of NSCLC may be different from that of other tumor types, these support our findings of long RTL in peripheral blood as a potential biomarker for increased risk of recurrence in early stage NSCLC.

The potential mechanisms for long telomere as a poor prognostic marker are not well-established in the literature but are thought to be complex, reflecting a combined effect of epigenetic regulation, cytokines and hormones.[31,32] We can speculate that longer telomeres are secondary to increased telomerase activity that stabilizes telomere length and are therefore less susceptible to apoptosis, potentially contributing to increased number of viable tumor cells. In addition, longer telomeres generally represent actively reproducing cells that are at an increased risk of acquiring tumor-causing mutations whereas short telomeres may induce cellular senescence.[33,34] High levels of telomerase activity are seen in 80% to 85% of all NSCLCs and are associated with advanced stages of NSCLC.[35,36] In addition, telomerase activity can be enhanced by various circulating cytokines[37-39] that were detected at higher serum levels in untreated lung cancer.[40,41] Our finding is also supported by a recent study by Robles-Espinoza and colleagues that reported a mutation in the protection of telomeres (POT1) gene that predisposes to development of familial melanoma that is strongly associated with longer telomeres.[42] The caveat to above hypotheses is that telomere pathway in leukocytes may not reflect what is occurring in tumor cells. We are limited by the assumption that some inherent telomere-related properties are shared with cancer cells. This assumption may be supported by a recently published work by Daniali and colleagues which demonstrated that telomere lengths strongly correlated between tissues.[43]

Our data also suggest that impact of longer telomere length on increased risk of recurrence was more pronounced in female and adenocarcinoma subgroups. This finding is consistent with our recently published work demonstrating that patients with adenocarcinoma had longer telomere length than healthy controls while patients with squamous cell carcinoma had shorter telomere length.[44] This is further supported by an independent study that demonstrated a pronounced association between telomere length and lung cancer risk in female adenocarcinoma cases.[45] Longer telomeres in females were reported in several other studies including a recent meta-analysis.[46-48] Estrogen may explain gender difference with respect to telomere length. In support of this, postmenopausal women with a history of long-term hormone replacement therapy had longer blood telomeres than postmenopausal women without hormone replacement therapy.[49] Estrogen can also influence telomere length by activation of the hTERT gene promoter and by posttranscriptional regulation of hTERT.[50,51] Furthermore, a recent prospective cohort study among women in China by Lan and colleagues reported that females with longer telomere length in peripheral white blood cells may have an increased risk of developing lung cancer consistent with our finding.[30] It is also intriguing that longer telomere length predicted increased risk of recurrence only in patients with adenocarcinoma. Telomere pathway may be one of the necessary steps for pathogenesis in adenocarcinoma that is molecularly distinct from other histology types. Kuhn and colleagues reported that relative telomere length significantly differs by histologic type in ovarian cancer.[52] Specifically, longer telomeres in clear cell ovarian carcinoma are associated with increased mortality suggesting that aberrations in telomere length may impact progression of tumor. A different study by Hirashima and colleagues demonstrated that forced elongation of the telomeres in cancer cells promoted their differentiation *in vivo*.[53] The resulting cells had longer telomeres and were capable of forming tumors in nude mice that surprisingly exhibited many duct-like structures, consistent with adenocarcinoma. We could speculate that long telomere length could potentially contribute to tumor recurrence by regulation of cancer cell differentiation, especially into adenocarcinoma subtype. It is also worth noting that smoking-related NSCLC despite borderline significance in Table 3 may be an important subgroup in which telomere length may have a predictive value for recurrence. Further mechanical studies are necessary to better understand our findings.

It may be worth noting that mutations in the epidermal growth factor receptor (EGFR) which predict increased sensitivity to the EGFR tyrosine kinase inhibitors in NSCLC occur more frequently in women and adenocarcinoma histology.[54-58] Several studies suggest potential associations between telomere biology and EGFR signaling pathway. Heeg and colleagues reported that EGFR overexpression induces activation of telomerase via PI3K / AKT-mediated phosphorylation.[59] Another study involving clinical specimens of human salivary gland carcinomas reported a significant correlation between telomerase activity and mRNA expression of EGFR.[60] Furthermore, epidermal growth factor (EGF) up-regulated telomerase activity in EGFR receptor-positive cells after the activation of telomerase reverse transcriptase (TERT) mRNA expression.[61] This suggests that EGF activates telomerase through the direct activation of TERT transcription. Even though there is no study, to our best knowledge, demonstrating relationship between telomere length and EGFR mutation status, it may warrant further investigation. Since most of our study subjects were enrolled

in the study prior to our knowledge about sensitizing EGFR mutations, EGFR mutation status in tumors of our patient population is not available.

The major strengths of our study include use of a large number of well-characterized early stage NSCLC patients with detailed clinical information, reliable assay for telomere length measurement and a prospective cohort design. Furthermore, our study adjusted for multiple potential confounding variables such as age, gender, ethnicity, stage, pack year, and treatment regimens. A major limitation of our study is that in contrast to studies involving metastatic NSCLC patients, our early-stage NSCLC population had relative low number of events, which resulted in relatively small sample sizes in some subgroups. Our findings will need to be validated ideally targeting specific subgroups (females, adenocarcinomas, etc.) and in an independent patient population before clinical use.

In conclusion, this is the first large-scale study evaluating impact of telomere length on predicting risk of recurrence after curative resection in early-stage NSCLC patients. Our data support long telomere as a predictor for increased risk of recurrence, especially in female patients with adenocarcinoma. Our findings will need to be validated in an independent patient population with a special focus in females and adenocarcinoma subgroups before being utilized in clinical practice. Nevertheless, leukocyte telomere length appears to be a promising biomarker that can modulate clinical outcome in early-stage NSCLC patients.

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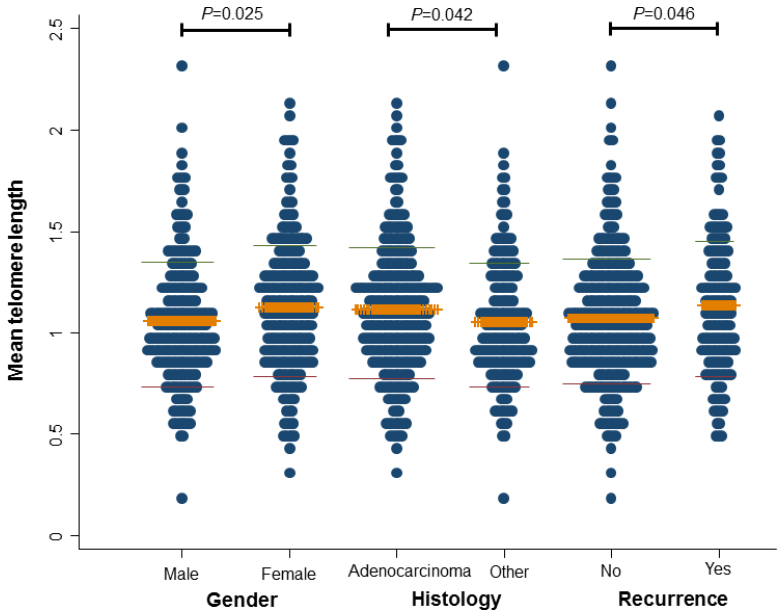

Figure 1. Association of telomere length with gender, histology and recurrence

Females had longer RTL compared to males, and the patients with adenocarcinoma demonstrated longer RTL compared to those with other histologic types. Patients who developed recurrence had significantly longer mean RTL compared to those without recurrence.

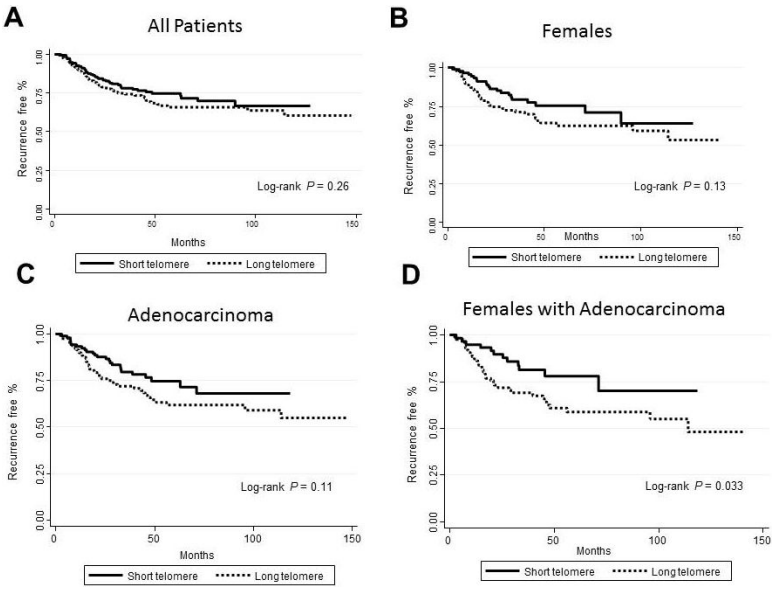

Figure 2. Kaplan-Meier estimates of recurrence in NSCLC patients with long versus short RTL following curative resection

In females with adenocarcinoma (D), those with longer telomeres had significantly longer time to recurrence.

Table 1

Patient characteristics

Median follow-up time(months)	61.02
Age, median(range)	66.0(29-86)
Total number of patients	473

Variables	N	%
Sex		
Male	232	49
Female	241	51
Ethnicity		
White	418	88.4
Black	35	7.4
Others	20	4.2
Smoking status		
Never	65	13.7
Former	241	51
Current	167	35.3
Clinical stage		
Stage IA	212	44.8
Stage IB	170	35.9
Stage IIA	24	5.1
Stage IIB	67	14.2
Pathologic stage		
Stage 0	2	0.4
Stage IA	185	40.4
Stage IB	164	35.8
Stage IIA	27	5.9
Stage IIB	80	17.5
Histology		
Adenocarcinoma	291	61.5
Squamous cell carcinoma	121	25.6
Other	61	12.8
Treatment		
Surgery only	332	73.3
Adjuvant Chemotherapy	89	19.6
Neoadjuvant Chemotherapy	32	7.1
Type of surgery		
Wedge resection only	52	11
Lobectomy only	310	65.5
Pneumonectomy only	23	4.9
Other	88	18.6

Table 2

Relationship between leukocyte telomere length and various clinicopathologic parameters

Variables	N	Mean (SD)	P-value
Age	473	1.09(0.32)	1.50E-08
Sex			
Male	232	1.06(0.31)	
Female	241	1.12(0.32)	**0.025**
Smoking status			
Never	65	1.14(0.36)	
Ever	408	1.08(0.31)	0.137
Recurrence			
No	322	1.07(0.31)	
Yes	151	1.13(0.33)	**0.0465**
Histology			
Adenocarcinoma	291	1.11(0.32)	
Others	182	1.05(0.30)	**0.042**
Ethnicity			
White	418	1.09(0.31)	
Others	55	1.11(0.34)	0.611
Stage			
1	382	1.09(0.32)	
2	91	1.09(0.30)	0.933
Treatment			
Surgery only	332	1.10(0.32)	
Surgery+Chemo	121	1.07(0.29)	0.28

Table 3

Multivariate Cox proportional hazard regression analysis of telomere length and recurrence in the overall and stratified analysis

Multivariate Analysis*	N	HR	95% lower	95% upper	P value
All patients	427	1.75	0.96	3.22	0.070
Age<=65	204	1.88	0.84	4.22	0.13
Age>65	223	1.52	0.58	3.98	0.40
Male	209	1.15	0.40	3.26	0.79
Female	218	2.25	1.02	4.96	**0.044**
White	381	1.65	0.87	3.16	0.13
Others	46	8.91	0.63	125.68	0.11
Never smoker	56	0.12	0.01	1.90	0.13
Ever smoker	371	1.88	0.99	3.57	0.054
Adenocarcinoma	262	2.19	1.05	4.55	**0.036**
Other histology type	165	1.10	0.34	3.56	0.87
Stage I	341	1.52	0.74	3.12	0.25
Stage II	86	2.53	0.76	8.48	0.13
Surgery only	287	1.90	0.86	4.23	0.11
Surgery +chemo	120	1.10	0.35	3.51	0.87
Female + Adenocarcinoma	163	2.67	1.19	6.03	**0.018**

Abbreviations: HR, hazard ratio

*Adjusted for age, gender, ethnicity, stage, pack year, and treatment regimens.