High Telomerase Activity in Primary Lung Cancers: Association With Increased Cell Proliferation Rates and Advanced Pathologic Stage

Juan Albanell, Fulvio Lonardo, Valerie Rusch, Monika Engelhardt, John Langenfeld, Wei Han, David Klimstra, Ennapadam Venkatraman, Malcolm A. S. Moore, Ethan Dmitrovsky*

Background: Telomerase enzyme activity is not detected in most normal cells, a phenomenon believed to be associated with limitations on cellular proliferation. Since this activity is detected in nearly all human tumors, including non-small-cell lung cancers, it has been suggested that telomerase activation may be coupled to acquisition of the malignant phenotype. In this study, we determined whether telomerase activity was associated with tumor pathologic stage, tumor cell proliferation rates, and clinical outcome in a cohort of patients with resected non-small-cell lung cancers for whom long-term follow-up was available. Methods: Primary tumor specimens from 99 patients treated with surgery alone and six patients treated with surgery after chemotherapy were analyzed. Telomerase activity was measured by means of a modified Telomeric Repeat Amplification Protocol (TRAP) assay. Southern blot analysis of terminal restriction fragments was used to evaluate telomere length. Immunohistochemical analysis of Ki-67, a proliferation-associated nuclear antigen, was used to assess tumor cell proliferation. Results: Telomerase activity was detected in 84 of the 99 tumors treated with surgery alone; this activity was not detected in specimens of adjacent, benign lung tissue. Telomerase was detected in only three of six tumors resected after chemotherapy. For the surgery-alone group, statistically significant positive associations were found between the level of telomerase activity and tumor stage, lymph node metastasis, pathologic TNM (tumor–node–metastasis) stage, and Ki-67 immunostaining; a statistically significant inverse association was found between telomerase activity and patient age. No statistically significant differences in telomere length were found in relation to telomerase activity or pathologic stage. Telomerase activity was not found to be associated with clinical outcome in a multivariate Cox proportional hazards analysis adjusted for tumor stage and lymph node status. Conclusions: High telomerase activity is detected frequently in primary non-small-cell lung cancers that exhibit high tumor cell proliferation rates and advanced pathologic stage. [J Natl Cancer Inst 1997;89:1609–15]

Human telomeres are specialized nucleoprotein structures located at the ends of chromosomes and are composed of tandem repeats of the sequence 5’-TTAGGG-3’ bound to specific proteins. Conventional DNA polymerases cannot replicate the ends of linear chromosomes, resulting in gradual telomere shortening when cells divide (1). Telomerase is a ribonucleoprotein that synthesizes de novo telomeric DNA onto chromosome ends, thus compensating for this ‘end-replication problem’ (2,3). In somatic cells, where telomerase activity is usually not detected, there is progressive telomere shortening during replication, and telomere length often reflects cellular proliferative potential (4–7). In contrast, germ cells and most immortalized human cell lines exhibit telomerase activity and stable telomere length (8–10). Telomerase activity is frequently expressed in human tumors, as assessed by the highly sensitive polymerase chain reaction (PCR)-based Telomeric Repeat Amplification Protocol (TRAP) assay (8). These findings, coupled with infrequent telomerase expression in normal cells, suggested that telomerase activation was tightly coupled to the acquisition of the malignant phenotype (8,11,12).

The RNA component of human telomerase (13) and a telomerase-associated protein have recently been cloned (14). These two components are widely expressed in human tumors and tumor cell lines, and they may be necessary for telomere elongation (13,14). In support of this possibility, treatment of immortalized human tumor cell lines with antisense oligodeoxynucleotides targeted to the RNA component of human telomerase led to a reinitiation of telomere shortening and induction of the massive cell death associated with proliferative senescence (13). The maintenance of a constant average telomere length in cells expressing telomerase activity seems to be regulated by a negative feedback loop. This idea is supported by functional studies with a recently cloned telomere-binding protein that suppresses telomere elongation and is proposed to be a negative regulator of telomerase activity (15).

Lung cancer remains the most common cause of cancer-related death in the United States for both men and women. A better understanding of the biology of lung tumors and identification of diagnostic markers or new therapeutic targets are urgently needed to develop novel treatment strategies that could improve the dismal survival rates of most patients with lung cancer (16,17). In a pivotal study (18), telomerase activity was found in 78.4% of non-small-cell lung cancers (NSCLCs) and in all small-cell lung cancers examined. Varying levels of telomerase activity were previously detected in NSCLCs (18), and it was hypothesized that the tumors with no or low telomerase activity might be composed primarily of mortal cancer cells. High levels of telomerase activity were detected in metastatic lesions even when undetected in the primary tumor, suggesting that telomerase activation contributes to the development...
of metastatic disease. This study (18) raised the prospect that telomerase might represent a marker for immortal lung cancer cells and a therapeutic target in lung cancer.

In our study, telomerase activity was measured by use of a recently modified TRAP assay (Kim NW, Wu F: personal communication) that increases the reliability of the assay, allows the expression of relative levels of telomerase activity, and identifies the presence of inhibitors of Taq polymerase. The purpose of this study was to analyze comprehensively whether a relationship existed between telomerase activity, tumor pathologic stage, tumor cell proliferation rates, and clinical outcome in a well-characterized cohort of patients with resected NSCLC for whom long-term clinical follow-up was available.

Methods

Tissue specimens. One hundred seven tissue specimens were obtained from consecutive patients with primary NSCLC who underwent potentially curative operations. Use of these tissue specimens was approved by the relevant institutional review boards. Protein extracts from 105 of these specimens were adequate for analysis. Pulmonary resection was accomplished by careful intraoperative staging with complete mediastinal lymph node dissection as described by Martini and Flehinger (19). Lymph nodes were labeled separately for pathologic analysis, in conformity with the American Thoracic Society Lymph Node Map (20). Within 10 minutes of completion of the pulmonary resection, a sample of the primary tumor, trimmed of the surrounding lung tissue and of any grossly necrotic material, was snap frozen in liquid nitrogen. A separate specimen of benign lung tissue was harvested from an area distant from the primary tumor and also frozen in liquid nitrogen. The presence of malignant or benign lung tissue in the harvested samples was confirmed by microscopic examination. An independent assessment by two pathologists (F. Lonardo and D. Klimstra) was performed, and both pathologists concurred with respect to the histopathologic features evaluated. The specimens were scored for tumor grade, proportion of tumor tissue present, proportion of necrosis in the tumors, and the estimated percentage of infiltrating lymphohematoid cells in areas with tumor. The tumor-node–metastasis (TNM) stage was determined according to the International Staging System for NSCLC (21).

Protein extraction. Frozen tissue samples (50–100 mg) were homogenized in 100–200 μL ice-cold CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate) lysis buffer (0.5% CHAPS, 10 mM Tris–HCl [pH 7.5], 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 5 mM β-mercaptoethanol, and 10 mg/mL leupeptine) with the use of disposable pestles and standard techniques. The tissue homogenates were incubated on ice for 30 minutes and then centrifuged at 12,000g for 30 minutes at 4°C. Supernatants were collected and stored at −80°C. Protein concentrations were measured by use of the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA), and aliquots containing 1 μg protein/mL were stored at −80°C (8,22,23).

TRAP assay. The telomerase assay was performed according to a recently modified TRAP assay protocol (Kim NW, Wu F: personal communication) (8,22–24). Extracts containing 2 μg of protein were assayed in reaction tubes that contained 50 μL of the TRAP reaction mixture. The TS primer was labeled at its 5′ end by use of 5 U T4 polynucleotide kinase (PNK; Promega Corp., Madison, WI) and 2.5 μCi of 3000 Ci/mmol [γ-32P]adenosine triphosphate per μg of TS. The kinase reaction mixture was incubated at 37°C for 20 minutes, and then the PNK was heat inactivated at 95°C for 5 minutes. Each TRAP reaction consisted of 5 μL 10x TRAP buffer (22), 50 μM standard deoxyxribonucleoside triphosphates, 0.1 μg end-labeled TS primer, 0.1 μg RP return primer, 0.1 μg NT internal control primer, 0.01 amol of the TSNT internal control template, 2 U Taq DNA polymerase (AmpliTaq; The Perkin-Elmer Corp., Branchburg, NJ), and the extract containing 2 μg protein. TSNT is an internal control PCR template amplified by the primers TS and NT, giving a 36-base-pair (bp) product. After a 30-minute incubation at room temperature, the TRAP reaction mixture was subjected to 30 cycles of PCR. The PCR products were resolved by electrophoresis in a 15% polyacrylamide gel under non-denaturing conditions, and the gel was analyzed on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). In every gel, the products of a negative control reaction (2 μL CHAPS lysis buffer) and of 0.1 amol of the quantitation standard oligonucleotide R8 were included. The telomerase quantitation results were expressed as total product generated (TPG) (see Fig. 1 for details of the quantitation). The specificity of the 6-bp ladders was confirmed by the absence of the ladders following heat inactivation of the protein extracts. All protein extracts were analyzed in at least two independent TRAP assays, and the average telomerase activity (TPG) was calculated (Fig. 1). Subgroups were defined as having negative (TPG = 0), low (TPG>0 and ≤5), moderate (TPG>5 and ≤30), or high (TPG>30) telomerase activity.

Alkaline phosphatase activity. Alkaline phosphatase activity was assayed as a control for possible protein degradation (23). Two tumor protein extracts had no detectable alkaline phosphatase activity and were not subsequently analyzed. Alkaline phosphatase levels were similar in extracts of normal and malignant tissues (data not shown).

Immunohistochemistry. Ki-67, a proliferation-associated nuclear antigen that is present only in proliferating cells (25), was assessed immunohistochemically to measure tumor cell proliferation rates. Five-micron-thick, paraffin-embedded sections were deparaffinized and rehydrated by use of standard techniques. Pretreatment of the sections consisted of digestion with 0.05% trypsin, followed by microwave wave treatment for 10 minutes. The sections were then exposed to 3% H₂O₂ for 5 minutes, saturated with 0.05% bovine serum albumin (BSA), and preincubated with normal horse serum (Cappel Research, Durham, NC) at a 1:20 dilution in 2% BSA.
phosphate-buffered saline (PBS) for 15 minutes at room temperature. The MIB-1 antibody (Immunotech, Westbrook, ME), used at a 1:50 dilution in 2% BSA–PBS, was applied at 4°C for 16 hours (25). The sections were then rinsed with PBS for 30 minutes, and a biotinylated anti-mouse immunoglobulin G (Vector Laboratories, Inc., Burlingame, CA) was applied at a 1:500 dilution in 1% PBS–BSA at room temperature for 60 minutes. The sections were then rinsed with PBS and incubated with peroxidase-conjugated streptavidin (Dako Corp., Carpinteria, CA) at a 1:500 dilution in 1% PBS–BSA at room temperature for 45 minutes. The sections were then rinsed with PBS for 30 minutes, and color from the chromagen diaminobenzidine (0.06% in PBS) was developed for 15 minutes. The sections were subsequently rinsed in water, counterstained with Harris-modified hematoxylin (Fisher Scientific Co., Pittsburgh, PA), rinsed in 1% acid alcohol for 2 seconds, rinsed in ammonia water for 15 seconds, dehydrated, and placed under coverslips in permount media. Ki-67 immunostaining was scored as the percentage of positive tumor cells per section.

**Terminal restriction fragment (TRF) length measurements.** For DNA isolation, tissues were incubated for at least 3 hours at 50°C with an appropriate volume of DNA extraction buffer (100 mM NaCl, 40 mM Tris [pH 8.0], 20 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate, and 0.1 mg/mL proteinase K), followed by phenol–chloroform–isoamyl alcohol extractions and precipitation with 3 M sodium acetate and ethanol. Electrophoresis of the undigested, high-molecular-weight DNA was performed to assess DNA degradation. Southern blot analysis to estimate telomere length was based on previously reported methods (4–7,26). Genomic DNA was digested with Msp I and Rsal I restriction endonucleases (Boehringer Mannheim, Mannheim, Germany) at 37°C for an appropriate length of time. Completeness of the DNA digestion was confirmed by means of gel electrophoresis; 10 μg of digested DNA was subjected to electrophoresis in 0.5% agarose gels. The resolved DNA was depurinated for 20 minutes in 0.2 M HCl, denatured for 30 minutes in 0.5 M NaOH–1.5 M NaCl, and neutralized for 30 minutes in 0.5 M Tris (pH 8.0)–1.5 M NaCl. The DNA was then blotted overnight onto nylon filters (Schleicher and Schuell, Inc., Keene, NH) in 20× solution of sodium chloride and sodium citrate (SSC). The filters were dried at 80°C for 1 hour and subsequently hybridized to a 32P end-labeled (32P) oligonucleotide probe (Genentech, La Jolla, CA) in a mixture that contained 5× SSC, 5× Denhardt’s solution, 10 mM phosphate buffer (pH 6.4), and 30 μg/mL salmon sperm DNA at 50°C overnight. The filters were washed twice in 0.5× SSC–0.1% sodium dodecyl sulfate for 15 minutes each at 50°C and then at room temperature for an appropriate length of time. To determine TRF length, the hybridized probe was visualized with a Phosphorimager (Molecular Dynamics), which quantified the radioactive signal in each of the lanes. Each lane was then graphically divided over the range of 2–23 kilobase pairs (kbp) into quadrants, and the densitometric counts in each quadrant were measured. The molecular-weight range of each quadrant was determined by use of radioactive markers. The mean and peak TRF lengths were calculated as described (26).

**Clinical database.** After pulmonary resection, the patients were seen in follow-up by one surgeon (V. Rusch), as previously reported (16). The parameters that were recorded included the patient’s age and sex, the tumor histology and stage, the estimated percentage of viable tumor cells present in the specimen, and the disease-free and overall survivals as calculated from the date of surgery. The telomerase activity, TRF length, and Ki-67 immunostaining results were evaluated without knowledge of the clinical outcomes.

**Statistical analysis.** Associations between telomerase activity (TPG) and the sex of the patient, tumor histology, lymph node metastasis, T stage, N stage, and pathologic TNM stage were evaluated by use of the Kruskal–Wallis test (27). Spearman’s rank correlations (27) were determined between telomerase activity and patient age, Ki-67 immunostaining, percentage of tumor cells in the specimen, percentage of necrosis in the specimen, and percentage of lymphocytic infiltration. The distribution of telomerase activity in relation to the degree of tumor cell differentiation was compared by use of the Wilcoxon test (27). Overall survival and disease-free survival were calculated from the date of thoracotomy by use of the method of Kaplan and Meier (28). The log-rank test (29) was used to compare survival across the subgroups of telomerase activity. All statistical tests were conducted at the two-sided, .05 level of significance. Proportional hazards regression was used to test the prognostic significance of factors in a multivariate model (30).

**Results**

**Telomerase Activity and Histopathology**

A total of 105 NSCLC specimens from 105 patients were examined in this study. Histologically benign lung tissue adjacent to 34 of these NSCLC specimens was also examined. Ninety-eight patients with stage I–IIIA tumors and one patient with stage IV disease were treated by surgical resection alone (Table 1); six patients had preoperative chemotherapy. The patients were treated during the period from January 1990 through May 1996, and clinical follow-up was updated as of September 1996. Telomerase activity was present in 84 (84.8%) of the 99 tumors from patients treated with surgical resection alone. Among these 84 tumors, 31 had low, 32 had moderate, and 21 had high telomerase activity (Fig. 1; data not shown). The linearity of the TRAP assay was confirmed over a three-logarithm (base 10) range of the target protein concentrations (Fig. 1, B). The average level of telomerase activity was 20 TPG (range, 0–134.9) (Fig. 2, A). Telomerase activity was not detected in 15 (15.2%) of the 99 tumors in addition to all adjacent, histologically benign lung tissue specimens examined (Fig. 1, A; data not shown). A histopathologic review of 81 of the tumors established that telomerase activity and the estimated percentage of tumor cells present in the specimen (P = .1), the proportion of necrosis in the specimen (P = .72), the degree of lymphocytic infiltration (P = .1), or the histologic grade of the tumor (P = .1).

**Table 1.** Comparison of tumor histology, primary tumor size, lymph node metastasis, and tumor stage with telomerase activity in primary, resected non-small-cell lung cancer*  

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of tumors</th>
<th>Negative</th>
<th>Positive</th>
<th>% positive</th>
</tr>
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<td>Adenocarcinoma</td>
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<td>30</td>
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<tr>
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<td>20</td>
<td>1</td>
<td>19</td>
<td>95.0</td>
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<tr>
<td>T2</td>
<td>66</td>
<td>13</td>
<td>53</td>
<td>80.3</td>
</tr>
<tr>
<td>T3</td>
<td>13</td>
<td>1</td>
<td>12</td>
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<td></td>
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<tr>
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<td>13</td>
<td>50</td>
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</tr>
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<tr>
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<td>15</td>
<td>84</td>
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</tr>
</tbody>
</table>

*See (21) for information on tumor staging.  
*The corresponding patient had a simultaneous solitary brain metastasis that was resected prior to thoracotomy.
Telomerase activity was not linked. Telomerase activity was not associated with the sex of the patient \( (P = .47) \) or the histologic tumor type \( (P = .87) \) (Table 1). Patient age was correlated inversely with telomerase activity \( (r = -0.4; \ P < .01) \). The remaining six patients had stage IIIA tumors that were treated by surgical resection after induction chemotherapy; the data from these patients were analyzed separately.

**Telomerase Activity and Tumor Cell Proliferation Rate**

The MIB-1 antibody and immunostaining were used to measure the tumor cell proliferation rates in 93 tumors. The percentage of Ki-67-positive tumor cells correlated with telomerase activity \( (r = +0.29; P < .01; \text{Fig. 2, B}) \). In telomerase-negative tumors, the average percentage of MIB-1 immunostaining tumor cells was 22%; for the telomerase-positive tumors, this percentage was 32%, 32%, and 49% for cases with low, moderate, and high telomerase activity, respectively.

**Telomerase Activity and Pathologic Stage**

Telomerase activity was detected in 95.0% of T1, 80.3% of T2, and 92.3% of T3 tumors (Table 1). The average telomerase activity (TPG) was 18 in T1 and T2 tumors compared with 36 in T3 tumors \( (P = .03) \) (Fig. 3, A, left panel). A significant association was found between telomerase activity and lymph node metastasis \( (N0 \ vs. N1-2, P = .05; \text{Fig. 3, A, middle panel}) \). Telomerase activity was detected in 79.4% of N0, 95.0% of N1, and 93.8% of N2 tumors (Table 1). The average telomerase activity (TPG) was 18.3 in N0 lesions, 16.2 in N1 lesions, and 32.7 in N2 lesions. Telomerase activity was associated with tumor pathologic stage, with average telomerase activities (TPGs) of 14.8, 18.2, and 30.2 in stages I, II, and IIa, respectively \( (P = .01 \text{ stage I versus stage II versus stage IIa; } P < .01 \text{ for stage I versus stages II–IIa; } \text{Fig. 3, A, right panel}) \).

Telomerase activity was detected in 78.2% of stage I, 94.4% of stage II, and 92.0% of stage IIa.

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**Fig. 2.** A) Total product generated (TPG), as a measure of the level of telomerase activity in primary, non-small-cell lung cancers, shows the continuum of the activity detected. B) The proliferative cell fraction was assessed by means of immunohistochemistry, using the antibody MIB-1, which recognizes the Ki-67 proliferation-associated nuclear antigen in paraffin-embedded tissue specimens. The percentage of Ki-67 staining tumor cells was correlated with telomerase activity (TPG) \( (r = +0.29; P < .01) \). See text for additional details.

**Fig. 3.** A) Telomerase activity (average total product generated [TPG] ± standard error of the mean [SEM]) was associated with tumor size and extension of the primary lesion (left panel, two-sided \( P = .03 \)), the presence of lymph node metastasis (middle panel, two-sided \( P = .05 \)), and pathologic TNM stage (right panel, two-sided \( P = .01 \)) in non-small-cell lung cancer. B) The Southern blot analysis of terminal restriction fragments (TRFs) in lung tumors. The tumor in lane 1 showed a long TRF (approximately 23 kilobases [kb]) and had high telomerase activity; DNA from matched, benign lung tissue was unavailable for this specimen. TRFs from representative tumor (T) and matched, histologically benign (N) lung tissue specimens are shown in lanes 2–13. Tumor mean TRF lengths were found to be similar to mean TRF lengths in matched benign lung tissue (compare lanes 2, 6, and 8 with lanes 3, 7, and 9, respectively), longer than mean TRF lengths in matched benign lung tissue (compare lane 4 with lane 5 and lane 10 with lane 11), or shorter than mean TRF lengths in matched benign lung tissue (compare lane 12 with lane 13). The sizes of molecular weight standards appear at the right of this figure. See text for additional details.
tumors (Table 1). One patient with stage IV (simultaneous solitary brain metastasis that was resected prior to thoracotomy) had a TPG of 108.

Telomerase Activity and Telomere Length

The mean TRF length ranged from 6.2 to 10.4 kbp in the 15 telomerase-negative tumors and from 5.5 to 23.0 kbp in the 30 telomerase-positive tumors examined (Fig. 3; B; data not shown). In telomerase-positive tumors, average mean TRFs were 7.5 kbp, 8.2 kbp, and 8.2 kbp in specimens with low, moderate, and high telomerase activity, respectively. No significant association between TRF length and tumor size, lymph node metastasis, or pathologic stage was found. In 34 cases, TRF analysis was performed in both the tumor and adjacent, histologically benign lung tissue. Similar mean TRFs were measured in 26 (76%) cases, but TRFs were reduced in the tumor in six (18%) cases and elongated in the tumor in two (6%) cases. Peak TRF values were similar between the tumor and benign lung tissue in 23 (68%) cases but were reduced in the tumor in eight (24%) cases and elongated in the tumor in three (9%) cases. Among the four telomerase-negative tumors, the TRF lengths were similar to those found in normal tissues in three cases and reduced in the tumor in one case. The average telomerase activities (TPGs) were 16, 13, and 18 in tumors with elongated, reduced, and unchanged TRF lengths, respectively.

Telomerase Activity and Clinical Outcome

There were no statistically significant differences in disease-free survival or overall survival between patients grouped on the basis of telomerase activity ($P = .1$; data not shown). The 3-year actuarial disease-free survival was 57% for patients with telomerase-negative tumors compared with 40% for patients whose tumors had high telomerase activity, but this difference was not statistically significant.

The prognostic significance of telomerase activity was measured by use of a Cox proportional hazards model. Since $T$ status and $N$ status are known prognostic factors in lung cancer, these parameters were included in the analysis. The hazards ratios (and 95% confidence intervals) for overall survival were 1.78 (1.23–2.56) for $T \leq 2$ versus $T > 2$, 0.66 (0.48–0.91) for $N = 0$ versus $N > 0$, and 1.07 (0.63–1.82) for telomerase-negative versus -positive cases. Similarly, the hazard ratios (and 95% confidence intervals) for disease-free survival were 1.77 (1.25–2.52), 0.61 (0.45–0.82), and 1.24 (0.74–2.08), respectively. No statistically significant association was found between telomerase activity and clinical outcome after this analysis.

Telomerase Activity in Stage IIIA NSCLC After Preoperative Chemotherapy

Telomerase activity was not detected in three of six primary stage IIIA tumors resected after induction chemotherapy (31) (data not shown). The three telomerase-negative tumors had a major pathologic response to chemotherapy. The remaining three tumors had detectable telomerase activity and a less marked response to neoadjuvant chemotherapy.

Discussion

This study extends previous work (18) by demonstrating that high telomerase activity in primary NSCLC is frequent in specimens with high cellular proliferation rates and is associated with tumors presenting with advanced stage. Telomerase activity was detected in 84.8% of the NSCLC specimens from patients who underwent surgical resection only. Telomerase activity was significantly higher in advanced-stage disease than in early-stage disease. Telomerase activity was inversely associated with age, as has been seen with at least one other tumor (32). Telomerase activity has been linked to tumor stage in previous studies of neuroblastoma (33), breast cancer (34), gastric cancer (35), and leukemias (36). However, such an association was not found in studies of renal cancer (37), breast cancer (32,38), gynecologic tumors (39), or hepatocellular carcinoma (40).

In this series, telomerase-negative tumors were infrequently associated with lymph node metastasis at presentation. This finding could relate to the need for extensive cell proliferation for metastasis to develop from individual clones. The clonal expansion required could lead to critically reduced telomere lengths in the absence of telomerase reactivation, limiting the potential for metastatic progression. In three of six stage IIIA NSCLC cases having major pathologic responses to chemotherapy, telomerase activity was not detected in the tumors after chemotherapy. Perhaps repressed telomerase activity results from effective chemotherapeutic treatment. Examining this possibility should be the subject of future work.

A modified PCR-based telomerase assay was used in this study. It is worth noting that some variables could have influenced the telomerase activity measurements. For instance, alkaline phosphatase measurements were used to assess the integrity of extracted protein. However, the stability of alkaline phosphatase activity may not parallel the stability of telomerase activity, which could be more sensitive to minor protein degradation in the extracts. The efficiency of protein extraction may also have varied between the analyzed tissue specimens. While the percentage of tumor cells present in adjacent tissues was scored, the percentage of tumor cells present in the specimens used for telomerase activity measurements was not scored, since the same tissue cannot be processed for both parameters.

A small proportion of human tumors does not exhibit telomerase activity (41,42). In our study, telomerase activity was not detected in 15.2% of the primary lung cancers treated by surgical resection alone. In a study of retinoblastoma, a developmental tumor with a limited number of associated mutations, telomerase activity was absent in 50% of the examined tumors (43). It is possible that the requirement for telomerase in tumorigenesis depends on the telomere lengths found in precursor cells and on the number of clonal expansions needed (41). Additional explanations for telomerase-negative tumors include the following: 1) telomerase activation followed by its repression after telomere elongation, 2) telomerase down-regulation associated with cellular quiescence, 3) telomerase activity below the level of detection of available assays, or 4) alternative mechanisms to compensate for the end-replication problem. The existence of an alternative pathway was reported in immortalized cell lines and was associated with very long telomeres (up to 50 kbp) (44). However, very long telomeres were not found in this study for the 15 telomerase-negative NSCLC speci-
mens examined for telomere length. Thus far, evidence of telomerase-negative clinical tumors having such long telomeres is not reported.

Few studies have comprehensively addressed the relationship between telomerase activity and prognosis. In neuroblastoma, high telomerase activity correlated with poor prognosis (33). Similar findings are reported in gastric cancer (35) and breast cancer (45) but not in renal cell carcinomas (37). In this series, NSCLCs having high telomerase activity had a more unfavorable prognosis than telomerase-negative tumors, but the differences were not statistically significant. While these data suggest that telomerase has a weak, or no, prognostic impact in lung cancer, perhaps the addition of more patients to this series and a longer follow-up would clarify the question of prognostic impact of telomerase activity in NSCLC. In this series, three patients with telomerase-negative primary NSCLC still relapsed despite surgical resection. Selection of telomerase-positive clones may eventually occur at distant metastatic sites when telomeres are critically shortened. It is reported in lung cancer that telomerase activation and telomere shortening at distant metastatic sites can occur when primary tumors are telomerase negative (18). Since most patients with lung cancer succumb to distant disease, anti-telomerase treatments may have a therapeutic role in the prevention of metastasis after successful local control of the primary tumors (9,46).

In many tumors, the mean telomere length, as estimated by TRF analysis, is similar to that found in the corresponding adjacent normal tissue (33,34,41,47,48). In this study, the mean and peak TRF lengths in the tumors were often similar to those in the adjacent, benign lung tissue, although the values were occasionally either smaller or larger in the tumor. Altered TRF lengths are reported to be linked both to high telomerase activity (33,34) and to the lack of measurable telomerase activity (37). In tumor-derived cell lines, no association was found between telomere length and telomerase activity (18). As recently reported, telomere length alone is unlikely to be an accurate predictor of cellular immortality (34).

Telomerase activity is linked to proliferation in diverse cellular contexts (26,49). In quiescent, primitive, hematopoietic progenitor cells, basal telomerase levels are low, but the enzymatic activity is rapidly up-regulated when the cells are activated to enter the cell cycle following exposure to combinations of hematopoietic growth factors (26). In certain human tumor cell lines, such as acute promyelocytic leukemia and human embryonal carcinoma cell lines, telomerase activity is repressed following induced differentiation in maturation-sensitive but not maturation-resistant cell lines (22,50). Telomerase is also repressed in tumor cell lines when the cells become quiescent, as reported previously (51,52). These observations indicate that telomerase activity and cellular proliferation may be linked in clinical tumors. In breast cancer, telomerase activity correlated with S-phase fraction in lymph node-positive breast cancer (45), but other investigators (32) failed to find such an association. In this study, a high proliferation rate for tumor cells was associated with telomerase activity, suggesting that telomerase is activated in lung cancer when growth-stimulatory signals are triggered. The pattern of telomerase activity versus Ki-67 immunostaining shown in Fig. 2, B, indicates that tumor cell subpopulations may exist, since specimens with no or low telomerase activity were less correlated with proliferation rates than specimens with higher telomerase activity (r = +.148 for activity ≤10 TPG and r = +.376 for activity >10 TPG, plus data not shown).

In summary, high telomerase activity measured in primary NSCLC was found to be associated with an increased cellular proliferation index and advanced tumor stage. These findings indicate that telomerase activity may contribute to lung tumorigenesis and its progression. These observations support the concept of telomerase as an attractive therapeutic target in lung cancer.

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Notes

Present address: J. Albanell, Oncology Service, Hospital Vall d’Hebron, Barcelona, Spain.

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