Telomerase Activity in Lung Cancer Cells Obtained From Bronchial Washings

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Background: **Telomerase, a ribonucleoprotein enzyme that functions in the maintenance of telomeres (specialized structures at the ends of chromosomes), has been reported to be a novel diagnostic marker for malignant diseases. We sought to determine whether measurement of telomerase activity in bronchial washings is of value in the diagnosis of lung cancer.** ***Methods:*** **Extracts of cells in bronchial washings were analyzed for telomerase activity by use of a telomeric repeat amplification protocol (TRAP) assay. Telomerase activity inside cells was evaluated by use of an *in situ* TRAP assay. The results of both TRAP assays were compared with those obtained from cytologic examination, which employed standard Papanicolaou staining.** ***Results:*** **When results from the two TRAP assays were combined, telomerase activity was detected in bronchial washings from 18 (82%; 95% confidence interval [CI] = 60%–95%) of 22 patients with lung cancer. In contrast, cancer cells were detected by cytologic examination in the bronchial washings of nine (41%; 95% CI = 21%–64%) of the same 22 patients, a statistically significant difference (two-sided *P* = .0061). In patients with lung cancer, telomerase-positive cells could be detected in bronchial washings irrespective of tumor location—11 of 14 (79%; 95% CI = 49%–95%) peripheral cancerous lesions and seven of eight (88%; 95% CI = 47%–100%) central cancerous lesions were detected by use of TRAP assays (for comparison, two-sided *P* = .5349).** ***Conclusions:*** **A high percentage of patients with lung cancers had detectable telomerase activity in bronchial washings. Thus, the use of a cell extract-based or an *in situ* TRAP assay in addition to cytologic examination may make the diagnosis of lung cancer more reliable. [J Natl Cancer Inst 1998;90:684–90]**

Telomeres are the very ends of chromosomes that, in mammalian cells, consist of long tracts of simple (TTAGGG) tandem repeats *(1).* Because of incomplete DNA replication at the chromosome ends, telomeres progressively shorten with each cell division in all somatic cells *(2–4).* Telomerase (a ribonucleoprotein enzyme) synthesizes telomeric DNA and can thereby completely or partially maintain telomere length *(5).* Introduction of a polymerase chain reaction (PCR)-based assay for telomerase (telomeric repeat amplification protocol; i.e., TRAP) *(6,7)* has made it possible to detect telomerase activity in approximately 85%–90% of primary cancer tissues *(8,9).* In contrast, in normal somatic cells, telomerase activity is usually undetectable *(6).* The few exceptions to this rule, in which telomerase activity is detected at low levels, include some hematopoietic cells *(10),* activated lymphocytes *(10,11),* hair follicle cells *(12),* intestinal crypt cells *(13),* basal cells of the epidermis *(14),* and proliferative phase endometrium *(15).* While it is still too early to conclude that telomerase will prove useful in cancer diagnosis or therapy *(16),* there are several generalizations that have emerged during the past year *(8,9).* For example, the telomere/telomerase hypothesis of cancer and the experimental results to date support the following: 1) Progressive telomere loss is normal in all somatic cells and may be the ''clock'' or timing mechanism that regulates cellular senescence; 2) modifications occur in the telomerase repression pathway, which causes the enzyme to upregulate (increase in amount) or reactivate (increase in activity); 3) telomerase activity in cancer cells correlates with the stabili-

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See ''Notes'' following ''References.''

zation of telomere length and cellular immortalization; and 4) telomerase activity or another mechanism that maintains telomeres is necessary for the continued proliferation of cells and is a critical, perhaps rate-limiting, step in cancer progression.

Lung cancer is one of the most common malignant diseases worldwide *(17)*. The diagnosis of lung cancer often requires many tests, including sputum cytology, chest x ray, computed tomography, and bronchoscopy *(17)*. Clinically, with the use of these methods, the early detection of lung cancer or the differential diagnosis of an abnormal shadow on chest x rays is often difficult, especially when cytologic examination fails to detect cancer cells. More importantly, the early detection of lung cancer or its differential diagnosis is clinically significant because some patients with early stage non-small-cell lung cancer are candidates for a surgical cure *(17)*. Clearly, other methods are needed to increase the reliability of the detection of early lung cancer.

Most lung cancer tissues, obtained during surgical resection of lung tumors, have telomerase activity when measured by the TRAP assay *(6,18,19)*. However, when the lesion is small or located peripherally, it is sometimes difficult to obtain appropriate tissue specimens for diagnosis. Transbronchial lung biopsies or brushings are often required to obtain lung tissues, but these techniques are often used for more advanced cases and are not very effective in the early detection of lung cancer. Considering these issues, we reasoned that we might be able to detect telomerase activity using bronchial washings that can be obtained during routine bronchoscopic examinations. Previously, telomerase activity has been detected in exfoliated cells of bladder cancer *(20–22)*, colon cancer *(23)*, and uterine cervical neoplasias *(24)*. Thus, using bronchial washings and a recently developed *in situ* TRAP assay *(25)*, we assessed telomerase activity in lung cancers at the cellular level and compared the data with the results obtained with an extract-based standard TRAP assay, referred to as a fluorescence-based TRAP assay in previous publications *(26,27)*. With these techniques, we sought to demonstrate the value of the combination of these two telomerase detection methods in the diagnosis of lung cancer.

Materials and Methods

Samples

With the use of a bronchofiberscope, bronchial washings were obtained from 22 untreated patients with lung cancer. Additional bronchial samples were obtained from patients without lung cancer (two old tuberculosis, two active tuberculosis, one emphysema, one pneumonia, five bronchitis, one hamartoma, two esophageal cancer without metastasis to the lung); and seven bronchial washing samples were obtained from individuals who had abnormal shadows, but who were not diagnosed as having lung disease. The diagnosis of the 22 patients with lung cancer was made by brush cytology or transbronchial lung biopsy. Of these 22 patients, 10 were treated with surgical resection and the remaining 12 (for whom surgery was not indicated) were treated with chemotherapy. The washings (20 mL of 0.9% sterile saline) were aspirated via gentle suction. The first aspirated sample was used for the standard cytologic examination, and the second suction was used for telomerase assays. Half of the second bronchial washing was used to detect telomerase activity by means of the extract-based TRAP assay, and the remainder was used for the *in situ* TRAP assay. The second bronchial washing was collected in a centrifuge tube (10 mL) and immediately cooled on ice and centrifuged at 1.500 rpm for 5 minutes at 4 °C. To avoid contamination with red blood cells that could potentially interfere with PCR, we subsequently resuspended the pelleted cells in 10 mL of filtrated hypotonic solution (1.2114 g Tris, 0.7456 g KCl, and 0.2033 g $MgCl_2$–$6H_2O$ to 1 L H_2O) and then pipetted the mixture 15 times to disperse the pellet. Immediately thereafter, we added 3 mL of filtrated hypertonic solution (16 g NaCl, 0.4 g KCl, 5.795 g Na_2HPO_4–$12H_2O$, and 0.4 g KH_2PO_4 to 1 L H_2O) *(28)*. Half of the suspended cells were centrifuged at 1.500 rpm for 1 minute at 4 °C, and the cell pellet was then stored at −80 °C until analyzed for telomerase activity by the extract-based TRAP assay (*see below*). The remaining half of the suspended cells was cytospinned for *in situ* TRAP assay (*see below*). We used COLO#320DM (a human colon cancer cell line) as a positive control.

Extract-based TRAP Assay

Telomerase activity was assessed according to the method of Kim et al. *(6)* and Piatyszek et al. *(7)* with modifications using the TRAPeze (*see* ''Notes'') detection kit (Oncor Inc., Gaithersburg, MD) *(29)* and an automated laser fluorescence DNA sequencer *(26,27)*; the TRAP assay procedure was performed according to the supplier's instruction and the report by Holt et al. *(29)*. The frozen cell pellets were dissolved in 10–30 μL of 1 × CHAPS, i.e., 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate lysis buffer, incubated on ice for 30 minutes, and then centrifuged at 10,000 rpm for 30 minutes at 4 °C. The supernatants were collected, and the protein content was determined by use of standard procedures (bicinchoninic acid protein assay). Briefly, 2 μL of the cell extract (equivalent 3 μg protein) was added to a 48–μL reaction solution consisting of 1 × TRAP buffer (Oncor Inc.), 1 × standard deoxynucleoside triphosphate mix (dNTPs) (Oncor Inc.), 10 pmol fluorescein isothiocyanate (FITC)-labeled TS primer 5′-AAT CCG TCG AGC AGA GTT-3′, 5′-end labeling using FluorePrime™ (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), TRAP primer mix containing a 36-base-pair (bp) internal standard (Oncor Inc.), 2 U *Taq* polymerase (Takara Shuzo, Shiga, Japan), and distilled water. The mixture was incubated at 30 °C for 10 minutes and then was heated at 90 °C for 90 seconds. The PCR conditions were 30 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1.0 minutes. The PCR products (1.5 μL) were subjected to 12% acrylamide denaturing electrophoresis in an automated laser fluorescence DNA sequencer II (Pharmacia LKB Biotechnology AB) and analyzed by the Fragment Manager program (Pharmacia LKB Biotechnology AB). Activity in the extract-based PCR TRAP assay was detected as a periodic 6-bp peak of telomerase products and, in each sample, the relative telomerase activity was calculated semiquantitatively *(26,27)* in comparison to a 36-bp internal standard (Fig. 1). Each specimen was analyzed at least twice and, in all instances, the replicated results confirmed the first analysis. To confirm that the sample contained telomerase activity, multiple periodic 6-bp peaks of telomerase signal had to be detected and preincubation of the extract with heat (95 °C, 1.5 minutes) or ribonuclease had to eliminate the periodic peak *(26,27)*. The multiple peaks corresponding to telomerase activity were inhibited by heat inactivation.

In Situ TRAP Assay

In situ PCR was performed as described previously *(25)*. Cells treated with hypotonic/hypertonic solution were cytospinned (400 rpm, 3 minutes) onto non-fluorescent silane-coated slide glasses and partially air dried. Twenty-five microliters of a solution containing 20 m*M* Tris–HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 63 m*M* KCl, 0.05% Tween 20, 1 m*M* EGTA, i.e., ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 50 m*M* dNTPs, 1 μg of T4 gene 32 protein (Boehringer Mannheim, Indianapolis, IN), bovine serum albumin (0.1 mg/mL), 2 U of *Taq* DNA polymerase, and 10 pmol FITC-labeled TS forward-primer (5′-AAT CCG TCG AGC AGA GTT-3′) was added to a chamber frame (15 mm × 15 mm) that had been placed over each partially dried specimen to hold reaction solutions, and the slides were incubated for 30 minutes at 22 °C. After TS extension, 25 μL of the same solution but with 10 pmol of FITC-labeled CX reverse-primer (5′-CCC TTA CCC TTA CCC TTA CCC TAA-3′) was added. The reaction chamber was sealed with a coverslip and heated to 90 °C for 1.5 minutes to inactivate telomerase. The telomerase reaction product was then amplified with the use of a Hybrid OmniSlide System thermocycler (National Labnet Co., Woodbridge, NJ). The PCR conditions were 30 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1.5 minutes. The slide chamber frames and top liners for the *in situ* PCR were removed completely, and the slides were washed in tap water, then sealed with a cover glass by use of MacIlvaine buffer/glycerin solution (1:1 = volume:volume). Cells were observed with a fluorescence microscope with a B-filter (Nikon, Tokyo, Japan). At least 100 cells of each specimen were examined at ×330 magnification, and cells with a fluorescence-positive nucleus were considered to be positive for telomerase activity. When a cell had brighter fluorescence in the cytoplasm than in the nucleus, it was considered to be negative for telomerase.

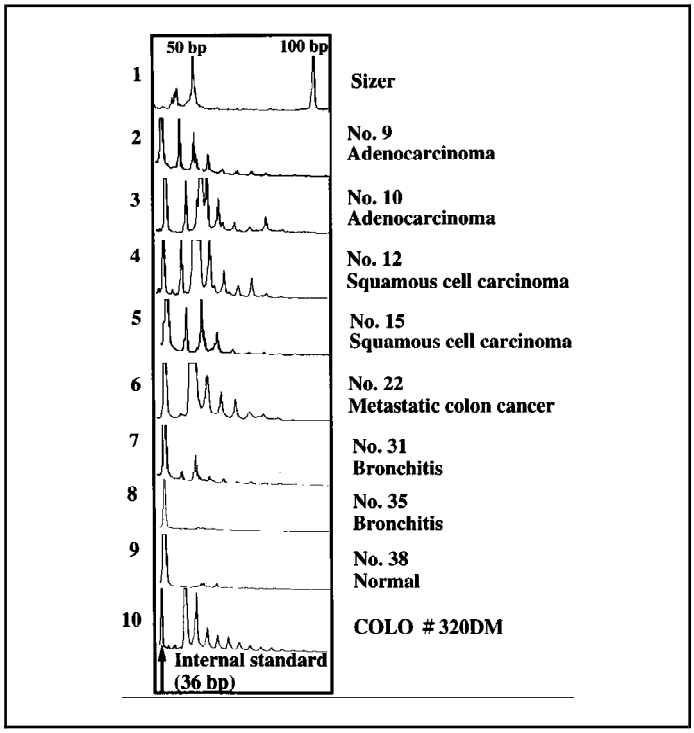

Fig. 1. Representative fluorocurves showing telomerase activity (telomerase signals). Each set of peaks corresponds to telomeric repeats that were synthesized by telomerase in bronchial washing cell extracts obtained from patients with lung cancer, as compared with activity in a human colon cancer cell line (COLO#320DM). To quantitate telomerase activity, we used an internal standard that shows a peak at 36 base pairs (bp). The results were analyzed by the Fragment Manager system, and the area of telomerase signals was compared with the area of the internal standard signal *(26,27).* In lane 1, the size markers (50 bp and 100 bp) are shown. Lane 2, patient 9 (adenocarcinoma, class IIIa cytology); lane 3, patient 10 (adenocarcinoma, class II cytology); lane 4, patient 12 (squamous cell carcinoma, class V cytology); lane 5, patient 15 (squamous cell carcinoma, class II cytology); lane 6, patient 22 (metastatic colon cancer, class IIIb: after heat inactivation [95 °C, 1.5 minutes] shows the inhibition of multiple peaks, indicating that these peaks correspond to telomerase activity); lane 7, patient 31 (bronchitis with infiltrating activated lymphocytes); lane 8, patient 35 (bronchitis without activated lymphocytes); lane 9, No. 38, bronchial washing sample from a normal individual; and lane 10, colon cancer line COLO#320DM.

Since the primers themselves are fluorescent, we performed *in situ* TRAP without PCR as a control. Other controls (data not shown) included using only one primer, PCR without primers, or PCR without *Taq* polymerase, as previously described *(25).*

Cytologic Examination

The cytologic examination of bronchial washings was performed by standard Papanicolaou staining. The various classes are defined as follows: class I, absence of atypical or abnormal cells; class II, atypical cytology but no evidence of malignancy; class III, cytology suggestive of, but not conclusive for, malignancy (IIIa, mild dysplasia; IIIb, advanced dysplasia); class IV, cytology strongly suggestive of malignancy; class V, cytology conclusive for malignancy *(30).*

Statistical Analysis

Comparisons of telomerase activity levels in groups were analyzed by use of the Mann–Whitney U test. We also used the chi-squared test and Fisher's exact test when appropriate. All P values are two-sided. Values of $P<.05$ were considered significant. Statistical tests were performed by use of the Statview (Brain Power Inc., Calabashes, CA) software package for the Macintosh personal computer. Microsoft Excel (version 5.0) was used to calculate 95% confidence intervals (CIs).

Results

Telomerase Activity in Cells From Patients With Lung Cancer Detected by the Extract-based TRAP Assay

In cells from bronchial washings, telomerase activity was detected in 16 of 22 (73%: 95% CI = 50–89%) untreated lung cancer patients (relative telomerase activity ranging from 0.91 to 6.24 in the 16 telomerase-positive patients). These included nine of 10 patients with adenocarcinomas, four of seven with squamous cell carcinomas, two of two with large-cell carcinomas, one of two with lung metastasis, and zero of one with small-cell carcinoma (Table 1). Levels (mean ± standard deviation) of relative telomerase activity in these telomerase-positive samples did not differ significantly among the various types of lung cancer; adenocarcinoma versus squamous cell carcinoma versus large-cell carcinoma = 2.58 ± 1.61 versus 2.85 ± 2.44 standard deviation (P = .6997) versus 1.51 ± 0.70 (P = .3458, determined by the Mann–Whitney U test), respectively.

Telomerase activity was detectable only in one of 12 specimens from patients with nonmalignant pulmonary disorders (Table 1). This telomerase-positive specimen was obtained from a patient with bronchitis (relative telomerase activity = 0.46), who exhibited infiltrating, activated lymphocytes. This specimen, in addition to one of two specimens from patients with nonmetastatic esophageal cancer, demonstrated lower relative telomerase activity levels when compared with those of specimens obtained from patients with lung cancers. Telomerase was not detectable in specimens obtained from any of seven normal individuals who were examined by use of the bronchoscope for abnormal x-ray shadows.

Telomerase Activity at the Cellular Level With the Use of an *In Situ* TRAP Assay

Seven patients with lung cancer had a high frequency (three or more cells per 330× magnification field) of bronchial-washing cells with bright fluorescence in the nucleus (Positive/Frequent in Table 1 and Fig. 2, A). Six of the seven samples had relative telomerase activity, ranging from 0.91 to 3.08, by use of the extract-based TRAP assay. Although the *in situ* TRAP detected a high frequency of cells with bright nuclear fluorescence in patient 11, the extract-based TRAP assay failed to detect telomerase activity. While the reason for this apparent discrepancy is not clear, it may be due to uneven distribution of telomerase-positive cells when dividing the specimen for the fluorescence TRAP and the *in situ* TRAP assays. Patient 15 had observable telomerase activity by use of the extract-based TRAP assay (relative telomerase activity = 1.23), while the *in situ* TRAP assay detected cells with only dim cytoplasmic fluorescence.

Eight lung cancer patients had bright nuclear fluorescence in fewer than three cells per 330× magnification field (Positive/Rare in Table 1 and Fig. 2, C). Seven of the same eight samples had telomerase activity determined by the extract-based TRAP assay, with telomerase activity ranging from 1.30 to 6.24. Of these eight patients, three showed aggregation of cells with bright nuclear fluorescence (Positive/Rare/Cluster in Table 1).

In patients with nonmalignant pulmonary disorders, zero of 12 had bright nuclear fluorescence (Table 1). Eight patients had

Table 1. Association between cytologic class and telomerase activity as determined by either fluorescence-based TRAP* assay or by an *in situ* TRAP assay in cells from bronchial washings

Diagnosis and patient No.	Age, y/sex†	Cytology (class)‡	Extract-based TRAP assay results§	*In situ* TRAP assay results‖	Cellular localization¶
Lung cancer					
1. Adenocarcinoma	73/M	V	0.95	Positive/frequent	Peripheral
2. Adenocarcinoma	66/M	V	2.3	Positive/rare	Peripheral
3. Adenocarcinoma	57/F	V	1.3	Positive/rare	Peripheral
4. Adenocarcinoma	49/M	V	3.08	Positive/frequent	Central
5. Adenocarcinoma	72/M	V	0	Negative	Peripheral
6. Adenocarcinoma	78/M	V	6.06	Negative	Peripheral
7. Adenocarcinoma	73/F	V	1.64	Positive/rare	Central
8. Adenocarcinoma	60/M	IIIb (atyp)	3.09	Negative	Central
9. Adenocarcinoma	66/M	IIIa	1.23	Positive/frequent	Peripheral
10. Adenocarcinoma	74/F	II	3.55	Positive/rare/cluster	Peripheral
11. Squamous cell carcinoma	71/M	V	0	Positive/frequent	Central
12. Squamous cell carcinoma	56/F	V	6.24	Positive/rare/cluster	Peripheral
13. Squamous cell carcinoma	68/F	IIIa (atyp)	0.91	Positive/frequent	Central
14. Squamous cell carcinoma	61/M	IIIa (atyp)	3.0	Positive/rare	Central
15. Squamous cell carcinoma	77/M	II	1.23	Cytoplasmic	Peripheral
16. Squamous cell carcinoma	84/M	II	0	Positive/rare/cluster	Central
17. Squamous cell carcinoma	58/M	I	0	Negative	Central
18. Large-cell carcinoma	51/M	II	2.0	Positive/frequent	Peripheral
19. Large-cell carcinoma	62/M	II (meta)	1.01	Positive/frequent	Peripheral
20. Small-cell carcinoma	52/M	II	0	Cytoplasmic	Peripheral
21. Metastatic renal cell cancer	65/F	II	0	Negative	Peripheral
22. Metastatic colon cancer	65/M	IIIb (atyp)	3.68	Positive/rare	Peripheral
Nonrespiratory cancers					
23. Esophageal cancer	67/M	II	0	Cytoplasmic	
24. Esophageal cancer	61/M	IIIa (meta)	0.78	Cytoplasmic	
Nonmalignant pulmonary disorders					
25. Old tuberculosis	74/M	I	0	Cytoplasmic	
26. Old tuberculosis	72/M	I	0	Cytoplasmic	
27. Tuberculosis	35/F	I	0	Negative	
28. Tuberculosis	67/F	II	0	Cytoplasmic	
29. Emphysema	62/F	I	0	Negative	
30. Pneumonia	66/F	I	0	Cytoplasmic	
31. Bronchitis	54/M	I	0.46	Cytoplasmic	
32. Bronchitis	68/M	II	0	Granular	
33. Bronchitis	60/M	II	0	Granular	
34. Bronchitis	55/F	II	0	Cytoplasmic	
35. Bronchitis	65/M	II	0	Cytoplasmic	
36. Hamartoma	36/F	II	0	Cytoplasmic	
Normal subjects					
37. Normal	53/M	I	0	Negative	
38. Normal	78/F	I	0	Negative	
39. Normal	42/F	II	0	Cytoplasmic	
40. Normal	71/F	II	0	Negative	
41. Normal	65/M	II	0	Negative	
42. Normal	57/M	II	0	Cytoplasmic	
43. Normal	64/F	II	0	Cytoplasmic	

*TRAP = telomeric repeat amplification protocol.

†M = male; F = female.

‡atyp = atypical cells; meta = metaplastic cells in the specimen.

§Relative telomerase activity in this assay was determined by use of the TRAP-eze kit and an automated laser fluorescence DNA sequencer (*see* ''Materials and Methods'' section for additional details).

‖Positive = positive cells for nuclear fluorescence; Cytoplasmic = cells with weak positive fluorescence in the cytoplasmic area without bright fluorescence at nucleus; Granular = cells with granular positive fluorescence in the cytoplasmic area; Negative = only background fluorescence. Frequent = three or more cells with bright fluorescence in the field of 330× magnification. Rare = less than three cells with bright fluorescence in the field of 330× magnification. Cluster = aggregrated cells with bright fluorescence.

¶Central = cancerous lesion was detected by a bronchofiberscopic examination; peripheral = cancerous lesion was not detectable by a bronchofiberscope.

cells with weak but uniform fluorescence only in the cytoplasm (Fig. 2, E), while two had granular, punctate fluorescence in the cytoplasm (Fig. 2, F).

Association Between Cytology and Telomerase Activity in Lung Cancer

Nine of the 22 samples (41%; 95% CI = 21%–64%) from lung cancers showed class V cytology upon Papanicolaou staining, indicating a high probability of malignant lung cancer (Table 1 and Fig. 2, B and D). Of the nine samples exhibiting class V cytology, only one (No. 5) did not show telomerase activity (Table 1). In 13 samples of lung cancer patients exhibiting class I–III cytology, three samples (Nos. 17, 20, and 21) showed no telomerase activity; the remaining 10 samples (77%; 95% CI = 46%–95%) had telomerase activity (Table 1). Thus, telomerase activity was detected in brochial washings from 18

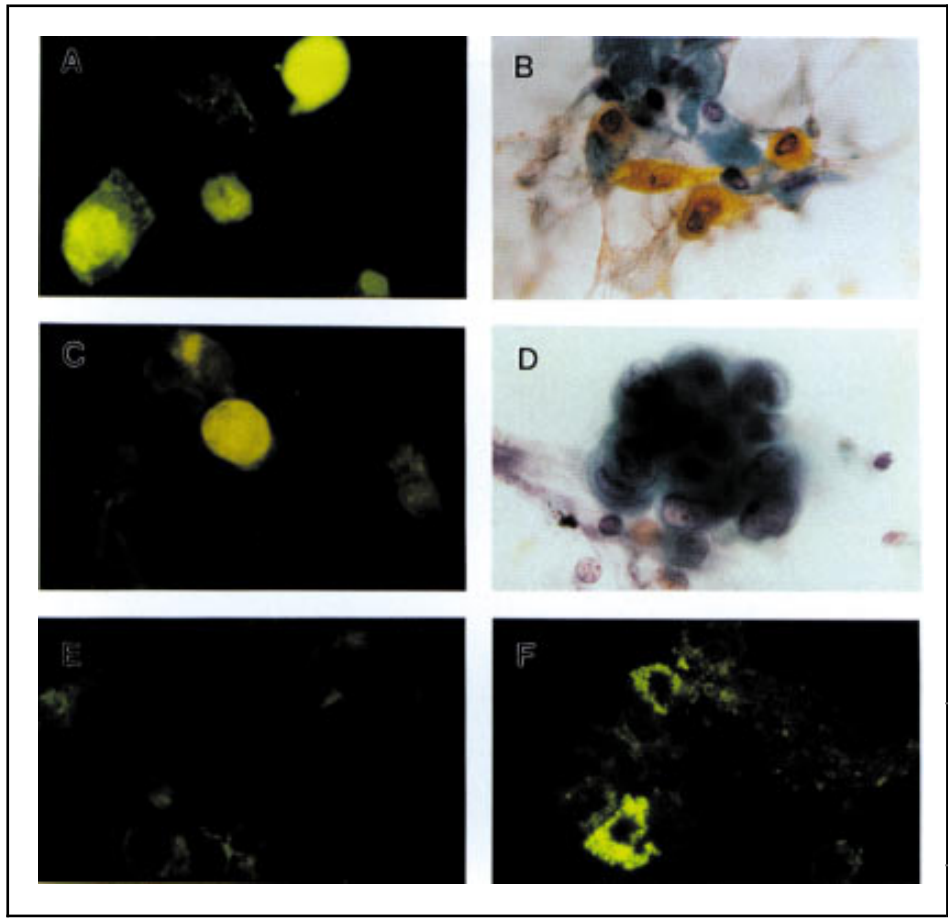

Fig. 2. Comparison of cytology and an *in situ* telomeric repeat amplification protocol (TRAP) assay of bronchial washings. Fluorescent signals corresponding to telomerase activity in cells from a bronchial washing obtained from a patient with lung cancer. Many bronchial washing cells (Positive/frequent in Table 1), obtained from a patient with squamous cell carcinoma (patient 11), had a bright fluorescence corresponding to telomerase activity (original magnification ×330, **A**) with class V cytology **(B).** In the bronchial washing sample obtained from a patient with adenocarcinoma (patient 3), a minor population (Positive/rare in Table 1) of bronchial washing cells showed bright fluorescent signals at the nucleus **(C)** and class V cytology **(D).** In patient 30 (pneumonia), only cells exhibiting a weak cytoplasmic fluorescence without a nuclear bright fluorescence were detected **(E).** Punctate fluorescence in the cytoplasm without a bright fluorescence in the nucleus was noted in the bronchial washing specimen obtained from a patient with bronchitis (patient 32) **(F).**

of 22 (82%; 95% CI = 60%–95%) lung cancer patients, but cancer cells were detected by cytology in only nine of 22 (41%; 95% CI = 21%–64%) of the same patients ($\chi^2 = 7.765$; $P = .0061$).

Association Between Localization of Tumor and Telomerase Activity

We also assessed the relationship between telomerase activity in bronchial washings and the site of the tumor (Table 1). Telomerase activity was detected in 11 of 14 (79%: 95% CI = 49%–95%) peripheral cancerous lesions and in seven of eight (88%; 95% CI = 47%–100%) central cancerous lesions (Table 2). This result indicates that by use of bronchial washings, telomerase-positive cells can be detected irrespective of tumor location ($\chi^2 = 0.273$; $P = .5349$). Also, the level of relative telomerase activity in telomerase-positive samples was not significantly different between central and peripheral tumors; central type versus peripheral type = 2.34 ± 1.01 versus 2.69 ± 1.96, respectively ($P = .8651$ determined by the Mann–Whitney U test).

Discussion

Telomerase activity is detectable with a high frequency in lung cancer tissues *(6,18,19).* Hiyama et al. *(18)* reported telomerase activity in 80% (109 of 136) of primary lung cancer tissues, while Yashima et al. *(19)* reported that 94% (32 of 34) of lung cancer specimens had detectable telomerase activity. In the present study, we demonstrated that 18 of 22 (82%; 95% CI = 60%–95%) bronchial washings from patients with lung cancer had telomerase activity by use of a combination of the extract-based TRAP and the *in situ* TRAP assay. Thus, telomerase detection in cells obtained from bronchial washings correlates well with the results obtained with the use of more invasive tissue biopsies. In some patients with lung cancer, the extract-based TRAP assay failed to detect telomerase activity. One possible explanation may be that these cancer cells did not contain telomerase activity. However, we cannot completely rule out the possibility of technical error or that perhaps some bronchial washings did not contain cancer cells. The current results confirm and extend previous reports *(6,18,19)* that high proportions of lung cancers have telomerase activity. More importantly, our results strongly suggest that bronchial washings might be used for the early detection of tumor cells, if they had telomerase activity, in potential lung cancer patients.

Of particular importance is that we could detect telomerase-positive cells in some patients whose cancers showed peripheral localization and class II/III cytology. If atypical or metaplastic cells in patients at risk for lung cancer contain telomerase activity and if this activity correlates with progression of disease, the detection of telomerase-positive cells could be of potential clinical value in the early detection of lung cancer. In support of this idea, it has recently been reported that some preneoplastic microdissected lung lesions contain weak, but detectable, telomerase activity *(19).* Taken together with our present observations and a recent report *(31),* this indicates that up-regulation of telomerase occurs early (preneoplasia) in the pathogenesis of lung cancer.

Table 2. Telomerase activity in bronchial washings from central versus peripheral lung cancers*

Cytology	No. of positive cases by extract-based TRAP	No. of positive cases by *in situ* TRAP	Total No. of telomerase-positive cases†	Coincidental rate of extract-based and *in situ* TRAP
Class I–III				
All patients	9/13 (69%)	8/13 (62%)	10/13 (77%)	10/13 (77%)
95% CI	39%–91%	32%–86%	46%–95%	46%–95%
Central type	3/5 (60%)	3/5 (60%)	4/5 (80%)	3/5 (60%)
95% CI	15%–95%	15%–95%	28%–99%	15%–95%
Peripheral type	6/8 (75%)	5/8 (63%)	6/8 (75%)	7/8 (88%)
95% CI	35%–97%	24%–91%	35%–97%	47%–100%
Class V				
All patients	7/9 (78%)	7/9 (78%)	8/9 (89%)	7/9 (78%)
95% CI	40%–97%	40%–97%	52%–100%	40%–97%
Central type	2/3 (67%)	3/3 (100%)	3/3 (100%)	2/3 (67%)
95% CI	9%–99%	29%–100%	29%–100%	9%–99%
Peripheral type	5/6 (83%)	4/6 (67%)	5/6 (83%)	5/6 (83%)
95% CI	36%–100%	22%–96%	36%–100%	36%–100%
Total				
All patients	16/22 (73%)	15/22 (68%)	18/22 (82%)	17/22 (77%)
95% CI	50%–89%	45%–86%	60%–95%	55%–92%
Central type	5/8 (63%)	6/8 (75%)	7/8 (88%)	5/8 (63%)
95% CI	24%–91%	35%–97%	47%–100%	24%–91%
Peripheral type	11/14 (79%)	9/14 (64%)	11/14 (79%)	12/14 (86%)
95% CI	49%–95%	35%–87%	49%–95%	57%–98%

*TRAP = telomeric repeat amplification protocol; CI = confidence interval.

†Number of telomerase-positive cases: combined data of extract-based TRAP assay and *in situ* TRAP assay.

Although telomerase activity was also detected in two samples without lung cancer, the relative telomerase activity in these noncancerous samples was low compared with that of lung cancer patients. One possible reason for detecting telomerase in these two specimens may be the presence of infiltrating activated lymphocytes that carry weak, but detectable, telomerase activity. Detectable telomerase activity in exfoliated cells from the urine *(21),* colon *(23),* and uterus *(24)* of noncancerous subjects was also reported in other diseases. Moreover, Yashima et al. *(19)* reported that approximately 20% of ''normal'' lung tissues of current smokers carry detectable telomerase activity.

Cytology is widely used for lung cancer screening, but a final diagnosis based on cytology alone is often difficult. It is noteworthy that, in the present study, 77% of patients with class I–III cytology had telomerase activity detected by a combination of the extract-based TRAP and the *in situ* TRAP assay. These observations illustrate one of the limitations of morphologic cytology: Since cytologic specimens contain many degenerated cells and may contain only a few cancerous cells, such specimens may hamper the proper cytologic diagnosis. Thus, using new diagnostic biomarkers such as telomerase in combination with cytology may prove more reliable in the diagnosis of cancer. Measurement of telomerase activity may be most helpful when cytologic examination fails to detect cancer cells. Assessing telomerase activity in cells from bronchial washings is usually easy and less invasive than obtaining tissue biopsy specimens.

Irrespective of tumor location, the detectability of telomerase activity and its magnitude were not significantly different (Table 2). Since central-type lung cancers are usually visible during bronchoscopy, pathologic diagnosis is easily obtained through a bronchofiberscopic biopsy. However, bronchofiberscopic biopsy is more difficult in peripheral-type lung cancer. Thus, our results demonstrating telomerase activity and telomerase-positive cells in bronchial washings may be useful in diagnosing peripheral-type lung cancers.

In conclusion, our results suggest that telomerase activity is detectable in bronchial washing cells. A combination of routine cytologic examination, an extract-based TRAP, and an *in situ* TRAP assay may improve the early diagnosis of lung cancer. Yet to be addressed is whether the current TRAP techniques may be useful in detecting minimal residual lung cancer following surgery and adjuvant therapy.

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Notes

K. Ohyashiki and J. H. Ohyashiki have applied for Japanese, United States, and European patents on the *in situ* TRAP assay. J. W. Shay holds stock in Geron Corp., Menlo Park, CA. The TRAPeze assay kit used in the study is technology sublicensed to the manufacturer (Oncor Inc.) by Geron Corp. No other research kits that use the technology of Geron Corp. were used in the study.

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