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Detection of telomerase activity in bronchial lavage as an adjunct to cytological diagnosis in lung cancer

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Abstract

Objective: Definitive diagnosis of lung cancer with conventional methods may sometimes be difficult in clinical practice. Telomerase is a ribonucleoprotein DNA polymerase that maintains the telomeric region of chromosomes during successive rounds of cell division. Telomerase activity in body cavity fluids has been advocated to be a potential diagnostic marker for malignancy. We investigated the diagnostic value of telomerase activity in bronchial lavage samples of patients undergoing diagnosis of lung cancer. **Methods:** A total of 29 bronchial lavage samples were collected from patients in whom the diagnosis was confirmed with cytological and/or histological examinations. Patients were classified as lung cancer patients (Group 1, $n = 22$) and patients with benign disease (Group 2, $n = 7$). Telomerase activity was determined with polymerase chain reaction-based TRAP (The telomeric repeat amplification protocol) assay. **Results:** Cytological examination was diagnostic in 12 (54.5%) of 22 patients in Group 1, and in all seven patients of Group 2 ($P = 0.063$). Telomerase activity was positive in 16 (72.7%) of Group 1 patients, while it was positive in only 1 (14.3%) sample of a lung abscess in Group 2 ($P = 0.011$). The sensitivity rate of cytological examination when combined with telomerase activity (81.8%) was significantly greater than that of cytological examination alone (54.5%) ($P = 0.031$). The sensitivity and specificity of telomerase activity were 72.7 and 85.7%, respectively. Telomerase activity had a positive predictive value as 0.94 and negative predictive value as 0.50. Diagnostic accuracy of telomerase activity was 75.8%. **Conclusion:** Telomerase activity in bronchial lavage is a highly sensitive diagnostic biomarker for malignancy and a potential complementary diagnostic technique to cytological examination in the diagnosis of lung cancer.

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Keywords: Telomerase; Lung cancer; Bronchial lavage

1. Introduction

Lung cancer accounts for the majority of cancer related death in men and women. Despite modern diagnostic and therapeutic advances, the 5-year survival rate following resection has only been improved in patients with early stages of disease. Thus, efforts have focused on early detection and intervention at an earlier stage to decrease the high mortality, which implies the significance of diagnostic methods in lung cancer.

The diagnosis and management of lung cancer is the most common indication for bronchoscopy in thoracic surgery. Various techniques such as forceps biopsy, bronchial brushing and bronchial lavage are used in conjunction with

bronchoscopy to provide specimens for either histological or cytological analysis. The cytological techniques used extensively in the diagnosis of lung cancer are bronchial lavage and bronchial brushing. Although previous studies showed the diagnostic complementary effect of cytological examination of bronchial lavage fluids [1,2], the diagnostic yield alone ranges between 24 and 64% [3]. Thus, it appears that a more efficient detection method such as a biochemical or immunologic marker may lead to a greater rate of diagnostic yield of lung cancer in bronchial lavage fluids.

Telomerase is a ribonucleoprotein DNA polymerase that compensates the telomeric loss by synthesizing telomeric DNA onto chromosomal ends, and it is believed to play an important role in the development of malignancy [4]. Telomerase expression has been detected in various human tumors, immortalized cell lines and at early stages of disease in breast and lung cancer [5]. Furthermore, telomerase activity has been shown to be a promising biomarker for the detection of lung cancer in malignant pleural effu-

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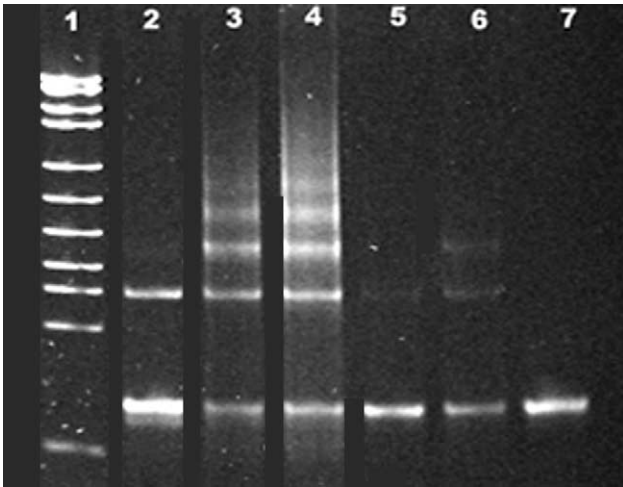


Fig. 1. Analysis of telomerase activity in benign and malign bronchial lavage fluids with TRAP assay. Lane 1, marker (pUC 18 Msp I, 26-SOLbp); lanes 2 and 3, telomerase (+) squamous cell carcinoma; lane 4, telomerase (+) small cell carcinoma; lane 5, RNase-treated squamous cell carcinoma; lane 6, telomerase (+) lung abscess; lane 7, negative control (CHAPS lysis buffer).

sions [4,6,7]. We conducted a study to outline the diagnostic value of telomerase activity for the detection of malignancy in bronchial lavage samples and compared the results with that of cytological examination.

2. Materials and methods

2.1. Patients and cytological samples

Bronchial lavage samples were collected from 29 patients who underwent diagnostic bronchoscopy in the Hospital of Kırıkkale University and in Ankara Oncology Hospital between January and June 2002. The patients were 21 men and eight women with a mean age of 56.2 ± 11.2 years (range, 27–73 years). Diagnosis was made on the basis of clinical and radiologic features, bronchoscopic findings, and cytological and/or histological examinations. Patients were classified into two groups. Group 1 consisted of 22 patients with lung cancer. Group 2 consisted of seven patients with benign disease who served as controls.

The patients were 16 male and 6 female with a mean age of 59.6 ± 9.0 years (range, 45–73 years) in Group 1. The tumor was endoscopically visible in 13 (59.1%) patients and invisible in nine (40.9%) patients. Squamous cell carcinoma (SCC) was the most common histological diagnosis in 12 (54.5%) patients followed by adenocarcinoma (AC) in eight (36.4%) patients and small cell carcinoma in two (9.1%) patients.

The patients were five males and two females with a mean age of 45.7 ± 11.8 years (range, 27–57 years) in Group 2. None of these patients had coexisting or previous cancer disease. Diagnosis was bronchiectasis in two patients, lung abscess in two patients, tuberculosis in one patient, pulmonary hamartoma in one patient, and esophageal diverticulum in one patient.

2.2. Determination of telomerase activity

The bronchial lavage samples were transported to the laboratory within 3 h of collection. After centrifugation at 2000 rpm for 10 min, the cell pellets were washed twice with ice-cold 10 mM PBS (phosphate-buffered saline, pH: 7.4) and treated with 200 μ l ice-cold CHAPS (3-[[3-chloromidopropyl]-dimethyl-ammonio]-1-propanesulfonate) lysis buffer. Following incubation on ice for 30 min, samples were centrifuged at $13\,000 \times g$ for 30 min at 4 °C. The protein concentrations of the supernatants were determined by the Bradford method [8].

Telomerase activity was determined with PCR (polymerase chain reaction)-based TRAP (telomeric repeat amplification protocol) assay as described previously [9]. Two microliters of supernatant (3 μ g protein/ μ l) was added to 43 μ l reaction mix containing 50 μ M dNTP, 0.1 μ g TS primer and 1 \times TRAP buffer. After incubation for 30 min at room temperature, 5 μ l amplifying mix containing 0.1 μ g CX primer and 2 U Taq DNA Polymerase (Epicenter) were added into PCR tubes. Telomerase products were amplified in 33 PCR cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s in a thermal cycler (Perkin Elmer 2400) with the hot start at 90 °C for 90 s.

Thereafter, 20 μ l of the PCR products were loaded on to a 12% polyacrylamide non-denaturing gel and resolved by electrophoresis at 200 V for 120 min in 0.5 \times Tris–borate–EDTA (TBE) buffer. The gels were stained with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene OR) for 30 min and visualized.

2.3. Evaluation of telomerase activity

The samples showing 6 bp-ladder bands were considered to be positive. To avoid the occurrence of false-positive results, 2 μ l extracts from all samples were treated with 1 μ g DNase-free RNase at 37 °C for 60 min prior to the TRAP assay and samples sensitive to RNase treatment were considered to contain telomerase activity. HeLa cells were used as positive controls, whereas CHAPS lysis buffer as negative controls (Fig. 1). Telomerase activity assays and cytological/histological examinations were performed independently in a blinded manner.

2.4. Statistical analysis

Results were analyzed with Fisher's exact test. The correlations in the malignant group were analyzed with the McNemar test. The following calculations were made: sensitivity = true-positive/true-positive + false-negative; specificity = true-negative/true-negative + false-positive; positive predictive value = true-positive/true-positive + false-positive; negative predictive value = true-negative/true-negative + false-negative; diagnostic accuracy = true-positive + true-negative/true-positive + false-negative + false-positive + true-negative. Data were expressed as mean \pm standard deviation (SD). A *P*-value of less than

Table 1
Results of cytological examination and telomerase activity regarding definitive diagnosis

	<i>n</i>	Diagnostic cytology No (%) of patients	Telomerase activity No (%) of patients
<i>Group 1 (lung cancer)</i>	22	12 (54.5)	16 (72.7)
Epidermoid carcinoma	12	8 (66.6)	10 (83.3)
Adenocarcinoma	8	4 (50)	4 (50)
Small cell carcinoma	2	–	2 (100)
<i>Group 2 (benign disease)</i>	7	7 (100)	1 (14.3)
Bronchiectasis	2	–	–
Lung abscess	2	–	1 (50)
Tuberculosis	1	–	–
Pulmonary hamartoma	1	–	–
Esophageal diverticulum	1	–	–

0.05 was considered statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences (SPSS, version 11.0, Chicago, IL, USA).

3. Results

Cytological examination was diagnostic in 12 (54.5%) of 22 patients in Group 1, and in all seven (100%) patients of Group 2 ($P = 0.063$) (Table 1). Telomerase activity was positive in 16 (72.7%) of Group 1 patients, whereas only 1 (14.3%) sample of a lung abscess (Fig. 1, lane 6) was found to possess weak telomerase activity in Group 2 ($P = 0.011$) (Fig. 2).

Out of ten cytologically false-negative lung cancer samples, six (60%) showed positive telomerase activity, whereas cytological examination was diagnostic in two (33.3%) of six lung cancer samples with negative telomerase activity ($P = 0.289$) (Fig. 3). Diagnostic yield reached up to 18 (81.8%) of lung cancer patients when cytological examination combined with telomerase activity, whereas 12 (54.5%) lung cancer patients could be diagnosed with cytological examination alone, which was a significant difference ($P = 0.031$).

The sensitivity and specificity of cytological examination were 54.5 and 100%, while the same rates were 72.7 and 85.7% for telomerase activity, respectively. Telomerase activity had a positive predictive value as 0.94 and negative predictive value as 0.50. Diagnostic accuracies of cytological examination and telomerase activity were 65.5 and 75.8%, respectively (Fig. 4).

Out of 13 visible tumors at bronchoscopy, nine (69.2%) were diagnostic by cytological examination of bronchial lavage samples, whereas three (33.3%) samples were diagnosed with the same method in nine invisible tumors ($P = 0.192$). Telomerase activity was positive in bronchial lavage samples of all 13 (100%) visible tumors, while only three (33.3%) showed positive telomerase activity among the nine invisible tumors ($P = 0.001$).

Cytological examination was diagnostic in eight (66.6%) of SCC and in four (50%) of AC. Similarly, the rate of telomerase activity in SCC was greater than that of AC. Out of 12 samples

with SCC, ten (83.3%) showed a positive telomerase activity, whereas only four (50%) samples with AC had a positive telomerase activity, however the difference did not show statistical significance ($P = 0.173$) (Fig. 5). Telomerase activity was positive in both of two (100%) small carcinoma samples, whereas cytological examination could not give a diagnostic yield in these samples.

4. Discussion

Telomeres are specialized structures which provide protective capping at the ends of the eukaryotic chromosomes, and this telomeric DNA consists of tandem repeated hexonucleotides TTAGGG in humans [10]. Telomeres shorten progressively with each cell division in all somatic cells resulting from incomplete DNA replication at the ends of the chromosomes. This mechanism is thought to be the 'biological clock' of the cell, which limits its reproductive capacity. In immortal cancer cells, telomeric DNA is synthesized by a ribonucleoprotein enzyme called telomerase that uses its own RNA component as a template and maintains telomere length during successive cycles of cell division [4,7]. Telomerase expression is thought to be a critical event responsible for carcinogenesis and a biomarker for cellular immortalization, which may indicate the proliferation of tumor cells.

Since the introduction of the original method [9], TRAP, which is a highly sensitive PCR-based assay, it became possible to detect telomerase activity with only a few malignant cells in the sample. TRAP is a cell and tissue extract based method that does not allow direct correlation between telomerase activity and cell type. Most human tumors express telomerase activity [11]; however, it is undetectable in normal somatic cells except proliferating cells of renewable tissues [12]. Although telomerase activity has also been investigated in lung cancer tissues [13–17], we are aware of only few reports clarifying the potential use of telomerase activity as a biomarker for malignancy with special reference to bronchial lavage samples [18–21]. These reports showed that the sensitivity of telomerase activity had a range of 68–82% in

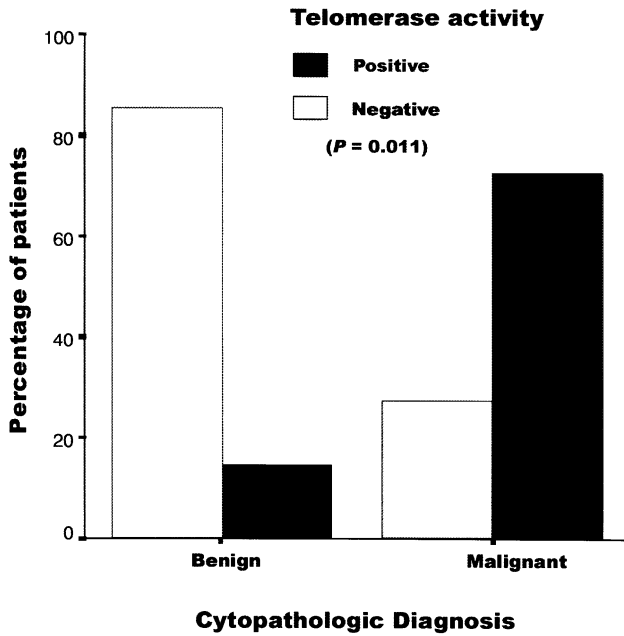


Fig. 2. Telomerase activity with respect to cyto/histologic diagnosis in groups.

malignant bronchial washings. Our data revealed the same rate as 72%, which falls between these ranges justifying the previous reports. Thus, the reported data indicate that telomerase activity in bronchial lavage samples is highly a sensitive diagnostic biomarker for lung cancer.

Our data revealed that the diagnostic yield of cytological examination showed a tendency toward significance between the groups, on the other hand, the rate of telomerase activity was significantly greater in lung cancer patients compared with that in patients with benign disease (*P* = 0.011). When we compared these diagnostic tools, we observed no signifi-

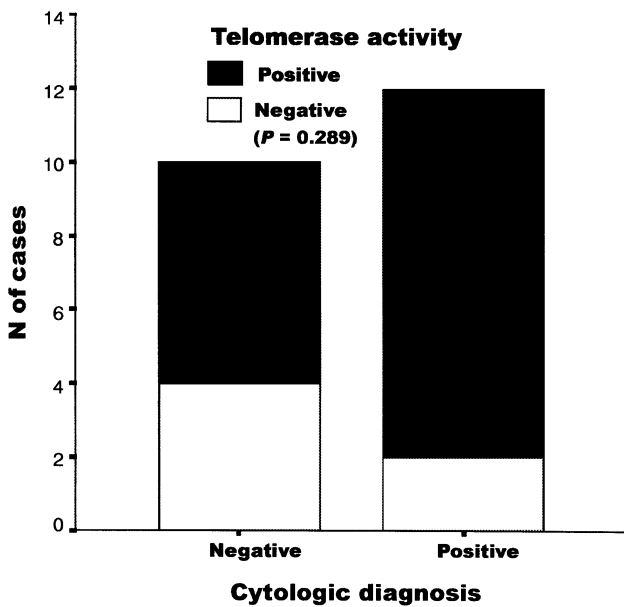


Fig. 3. The correlation of telomerase activity and cytological examination in patients with lung cancer.

cant correlation between the false negative samples of cytological examination those were diagnosed with telomerase activity to be malignant, and the opposite, false-negative cases of telomerase activity those were diagnosed with cytological examination (*P* = 0.289). Likewise, previous reports had similar data showing that no correlation existed between cytological examination and telomerase activity [19,20]. Thus, we may reasonably suggest that the detection of telomerase activity in bronchial lavage samples may provide a greater rate of definitive diagnosis for malignancy compared with cytological examination; however, neither of these methods are superior to each other. On the other hand, the sensitivity rate of cytological examination when combined with telomerase activity appeared to be significantly greater than that of cytology alone (*P* = 0.031), which implies the potential complementary role of telomerase activity in the diagnosis of lung cancer. This finding has also been noted in the previous reports [19,20].

The rate of false negative samples for telomerase activity ranged between 18 and 31% [18–21]. Our data revealed the same rate as 27% consistent with these reports. This finding may be attributed to several factors. Taq polymerase inhibitors such as hemoglobin, mucin, RNase and protease may take part in the medium with a resultant negative telomerase activity [11]. In addition, the lack of adequate number of cells extracted from the sample, and inactivation of telomerase enzyme during the freezing and thawing procedures may result in a negative telomerase activity [22].

Benign somatic cells usually do not express telomerase, however telomerase activity has been demonstrated in normal leukocytes and hematopoietic stem cells, proliferative cells of renewal tissues such as intestinal crypts, basal cells of the skin, hair follicles, and endometrial cells [12,23]. Likewise, the false-positive rate of telomerase activity occurred as 10% in some studies [6,24]. The specificity of telomerase activity appeared as 85% in our study. Among the 7 benign bronchial lavage samples, one (14%) with a lung abscess showed telomerase expression. The intense

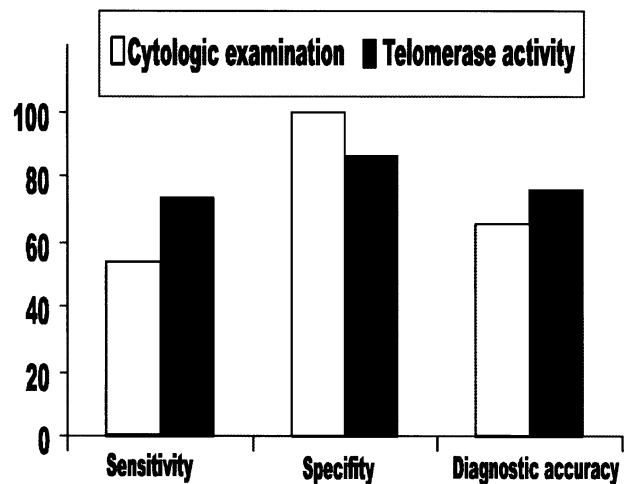


Fig. 4. Diagnostic values of cytological examination and telomerase activity.

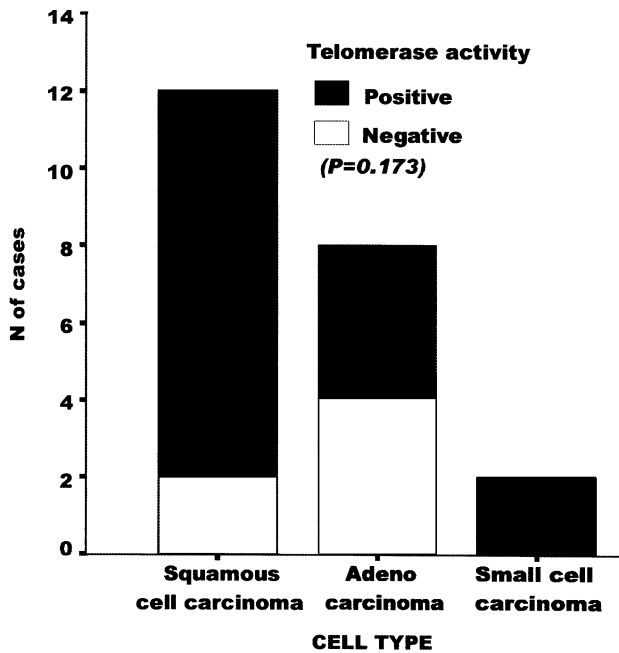


Fig. 5. Telomerase activity regarding cell type in the lung cancer group.

infiltration of activated lymphocytes and leucocytes in inflammatory processes might explain as to why this sample showed false-positive telomerase activity [12,25].

Although the difference was not significant, we observed a greater rate of telomerase activity in samples with squamous cell carcinoma when compared with adenocarcinoma cases, which was in line with a previous report [19]. This finding may be attributed to the more central localization of squamous cell carcinomas compared with adenocarcinomas and thus being more efficiently sampled with bronchoscopy. On the other hand, both of small cell carcinoma samples were positive for telomerase activity. Similarly, Hiyama et al. [13] showed that small cell carcinomas expressed higher levels of telomerase activity in human lung cancer tissues with a rate of 100% compared with that of non-small cell carcinomas, which was 80%.

In conclusion, the assay of telomerase activity in bronchial lavage fluids is highly sensitive in the detection of lung cancer. Telomerase activity has a higher diagnostic yield than cytological examination, and the diagnostic rate of bronchial lavage fluids will significantly increase when telomerase activity is used in conjunction with cytological examination as a complementary diagnostic tool.

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Appendix A. Editorial comment

Detection of telomerase activity in bronchial lavage as an adjunct to cytological diagnosis in lung cancer

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In the early years of the 20th century surgical resection, which was the standard form of treatment for most tumours, could not be applied to lung cancer due to the pneumothorax issue [1]. With this problem solved, surgical intervention became the treatment of choice at least since 1933 when the first pneumonectomy for cancer was performed [2]. One of the most significant advances in surgical therapy for lung cancer was the realisation in the 1950s that resectability did not equate with operability and that a selection process should operate in order, not only to reduce the number of thoracotomies which failed to offer successful resection but also to improve survival. This led to the establishment of oncologically based patient selection criteria for operation and monitoring of outcome and reliance on TNM classification. Progress in genetic and molecular biological research over the past 10–15 years is gradually shifting the emphasis from cyto/histopathological analysis to a more molecular based methodology for diagnosis and prognosis. It is now generally agreed that lung carcinogenesis is a multistep process and that genetic alternations precede morphological and cyto/histological changes. In recent years considerable effort has led to the discovery of a number of relevant proto-oncogenes, suppressor genes and DNA repair genes which are expressed aberrantly in lung cancer. The pressing issue is to establish the correlation between molecular genetic events and cyto/histological findings, which remain the gold standard of cancer diagnosis, and to identify biomarkers of cancer. An understanding of the genetic and molecular events will become increasingly important to the thoracic surgeon since, in the not-too-distant future, these are likely to be the tools of choice for detection of lung cancer in its early stages and for treatment monitoring and prognosis.

Currently, most research is directed towards study of the correlation between molecular events and histopathology and in identifying the markers which could signal genetic changes

in the cell from normal to malignant prior to microscopic cyto/histological manifestations. It is in this context that the study by Dikmen and colleagues [3], on detection of telomerase activity in bronchial lavage of lung cancer patients compared with those with benign lung disease, is of interest. The number of patients in the series is small and, therefore, it is difficult to draw decisive conclusions despite the rather strong statement by the authors that “assay of telomerase activity in bronchial lavage fluid is highly sensitive in the detection of lung cancer”. Dr Dikmen and colleagues’ study concerns 29 patients: 22 with malignant lung tumours and seven with benign disease. The aim was to outline the value of telomerase activity for detection of malignancy in bronchial lavage samples and to compare the results with that of cytology examination.

The rationale of the authors in comparing telomerase activity in malignant and benign disease is understandable, although the use of histologically normal tissue also would have been ideal. The fact that telomerase activity was observed in one case where the patient had only a lung abscess would support the use of normal control tissue. There are a few points emerging from this study which we believe to be important and could be relevant to cancer detection in conjunction with fluorescence bronchoscopy. We note that telomerase activity was recorded in all 13 patients whose cancers were visible at bronchoscopy. Ten of 12 patients with squamous cell cancer were telomerase-positive whereas only four out of eight patients with adenocarcinoma showed activity. These observations could suggest that telomerase activity as a marker is more likely to be of help in those cancers that arise in bronchial mucosa (central tumours) rather than in the periphery of the lung. The authors have not stated how many of these eight tumours were visible at bronchoscopy, but we assume from Table 1 that they would mostly have been peripheral tumours.

From the above observations one may postulate that telomerase could be a good tumour marker in bronchogenic squamous cell carcinoma, probably central as opposed to peripheral. This seems to accord with the preliminary results of a study that is in progress at the Yorkshire Laser Centre [4]. In this study high-risk volunteers are screened by white and fluorescence bronchoscopy; brush/biopsy sampling of bronchial mucosa; cyto/histology examination; and for telomerase activity. In our initial evaluation there was substantially higher activity in patients with abnormal fluorescence image of the mucosa than in those with a normal image. Clearly, therefore, larger definitive studies are needed to validate the promising results for the use of telomerase activity as a marker of tumour detection.

Overall, the results reported here are consistent with previous studies, cited by Dikmen and colleagues, demon-

strating that telomerase may have a useful role in the early diagnosis of lung cancer, as well as other tumours: a topic recently reviewed by Hiyama and Hiyama [5]. Reports on the clinical utility of telomerase are appearing more often in the literature, e.g. a recent study by Wang et al. [6] reported hTERT expression being associated with shorter overall survival, shorter disease-specific survival and shorter disease-free survival in stage I non-small cell lung cancer. In this study the independent prognostic value of TERT expression was confirmed in multivariate analysis.

Finally, as interesting as this study by Dr. Dikmen and colleagues is, it cannot support the conclusion that “assay of telomerase activity in bronchial lavage fluid is highly sensitive in the detection of early lung cancer” when telomerase measurements alone are used for diagnostic purposes, without the support of other genetic testing or fluorescence bronchoscopy. More evidence is needed before such a conclusion is justifiable, although it may not be too long before this is presented.

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