Telomerase Activity as a Prognostic Indicator in Stage I Non-Small Cell Lung Cancer[1]

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ABSTRACT

Patients with stage I non-small cell lung cancer (NSCLC) are typically treated with surgical resection alone. However, about one-third of such patients develop disease recurrence and die within 5 years after complete resection. The ability to predict recurrence could represent an important contribution to treatment planning. This study evaluates the presence of telomerase activity in tumor cells as a predictor of disease recurrence and cancer-related death after operation for stage I NSCLC patients. The activity of the telomerase enzyme was investigated by telomeric repeat amplification protocol (TRAP) in tumors and matching normal lung tissue samples obtained from 107 consecutive operable patients with pathological stage I NSCLC. Telomerase activity was detected in 66 (62%) of the 107 tumors examined and in none of the corresponding adjacent noncancerous lung tissue samples. Correlation with pathological parameters showed that telomerase activity was associated with histopathological grade ($P = 0.0135$) but not with tumor size or histological type. Univariate survival curves, estimated using the method of Kaplan and Meier, defined a significant association between telomerase activity and both disease-free survival ($P = 0.0115$) and overall survival ($P = 0.0129$). In multivariate analyses, performed by Cox's proportional hazards regression models, the presence of telomerase activity was the only strong predictor of disease-free survival ($P = 0.0173$) and overall survival ($P = 0.0187$). Our data indicate that telomerase activity can be an important prognostic factor that should be considered in future prospective trials of adjuvant therapy for high-risk stage I NSCLC patients.

INTRODUCTION

NSCLC[3] has become the main cause of cancer-related death in both men and women in western countries. Surgical resection represents the therapy of choice when the disease is localized. After surgery, patients with a more favorable prognosis are those with pathological stage I diseases ($T_{1-2} N_0 M_0$). The average 5-year survival rate for patients with stage I NSCLC is 65% (range, 55–72%; Refs. 1, 2). This means that about 30–40% of stage I patients will experience disease recurrence and die despite surgical resection. Therefore, new prognostic factors are needed to identify the subset of stage I patients likely to have recurrence, to treat them with a selective and effective adjuvant therapy.

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric repeats onto chromosomal ends using a segment of its RNA component as a template. It compensates for progressive telomere erosion that would otherwise occur in its absence (3–5). Studies of human tumors and human tumor cell lines indicate that telomerase activation may play a critical role in tumor cell growth by sustaining cellular immortality (6–8).

The highly sensitive PCR-based TRAP assay has allowed the identification of telomerase activity in most human malignancies (6, 9–11). Telomerase activity has been shown to correlate with poor clinical outcome in neuroblastoma, in gastric and breast carcinomas, and leukemia but not in renal cell tumors (12–17). In a recent study, Albanell *et al.* (18) report that NSCLCs having high telomerase activity have a more unfavorable prognosis than telomerase-negative tumors; however, the difference observed was not statistically significant. The screening of additional patients with a long follow-up could clarify the prognostic impact of telomerase activity in NSCLC. Moreover, because telomerase activity was found to be positively associated with pathological markers of poor prognosis, especially with the metastatic diffusion to thoracic lymph nodes (19), it is important to evaluate the prognostic meaning of telomerase activity in node-negative NSCLC patients, to eliminate the confounding influence of positive lymph nodes.

In the present study we assessed telomerase activity in a large series of NSCLCs from patients with stage I disease and correlated the results with pathological and clinical parameters. Our findings indicate that positive telomerase activity is strongly associated with prognosis in stage I NSCLC patients.

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; TRAP, telomeric repeat amplification protocol; CHAPS, 3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate.

MATERIALS AND METHODS

Patients and Samples Collection. The tumors for this study were obtained from a series of 118 stage I ($T_{1–2}$ N_0 M_0) NSCLC patients who underwent thoracic surgery during the period 1991–1993 at the Department of Surgery, University of Pisa. The study was conducted on 107 of these patients for whom complete follow-up data were available. Patient stage at the time of diagnosis was based on the international staging system for lung cancer (20). In each case, tumor and macroscopically normal lung tissue samples (taken as far as possible from the neoplastic area) were snap-frozen in liquid nitrogen within 10 min of excision and stored at $-80°C$. Immediately adjacent pieces of tumor and normal tissue were fixed and processed for light microscopy. All of the macroscopically normal samples were judged to be benign by histopathological examination.

Follow-Up. Follow-up data of the study population were obtained by direct patient contact. Collection occurred at 2-month intervals for the initial 2 years and at 4-month intervals after that. Recurrences were detected by pathological findings using bronchoscopic or bioptic specimens and a computed tomography scan or scintigram. Patients were categorized as alive with evidence of disease, alive without disease, and dead as a result of lung carcinoma. No patient in this series had died of cancer-unrelated cause. Time in days from the date of the operation to the date of follow-up or death was recorded.

Telomerase Assay. Frozen tissue samples (50–100 mg) were homogenized with a pestle in 100 μl of ice-cold CHAPS lysis buffer (0.5% CHAPS, 0.1 mM benzamidine, 10 mM TRIS/HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, and 5 mM β-mercaptoethanol). After maintenance at 4°C for 30 min, the lysate was centrifuged at $12.000 × g$ for 20 min at 4°C. The supernatant was removed, and its protein concentration was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munchen). The supernatant fluid samples were diluted to a concentration of 0.3 μg/μl with lysis buffer and stored at $-80°C$. Telomerase activity was assayed by using the Oncor TRAP-eze telomerase detection kit (Oncor; catalogue no. S7700). This kit represents a modification of the TRAP assay developed by Kim *et al.* (14). The procedure was performed according to the manufacturer's protocol. Briefly, a master mix containing all of the reagents outlined below except the protein extract was made for a final volume of 10 μl in each assay. The reagents included 10× TRAP buffer (1 μl), 50× deoxynucleotide triphosphates mix (0.2 μl), ^{32}P-labeled TS primer (0.4 μl), TRAP primer mix (0.2 μl), 2 units/μl Taq polymerase (0.08 μl), and distilled H_20 to a final volume of 10 μl. The TS primer was labeled at its 5′ end with 5 units of T4 polynucleotide kinase (Boehringer Mannheim Biochemica) and 2.5 μl of 3000 Ci/mmol [γ-^{32}P]ATP per 1 μg of TS. Heat-inactivated and test extracts (500 ng) were added to each tube. Heat inactivation was done by incubating 10 μl of each extract at 85°C for 10 min and adding 2 μl into each of the heat inactivation tubes. Control cell extracts containing telomerase (30 ng) was added to the positive control tube. All of the tubes were placed in a termocycler block and incubated at 30°C for 30 min. The samples were then subjected to PCR for 27 cycles of 30 s at 94°C and 30 s at 60°C. Five μl of the reaction were analyzed by electrophoresis on a 10% polyacrilamide gel under nondenaturing conditions. The gels were dried and exposed overnight on Kodak XAR films (Eastman Kodak Co., Rochester, NY).

Statistical Procedure. The different variables of the tumors analyzed were tested for association using the $χ^2$ and Fisher's exact tests. Overall survival was estimated by the method of Kaplan-Meier (21), and differences between curves were tested for statistical significance with the log-rank test (22). Cox's proportional hazards regression models (23) were used to assess the independent prognostic contribution of clinicopathological variables. The statistical analysis was performed using the Statview 4.5 statistical software run on a PowerPC G3 Macintosh computer.

RESULTS

Clinicopathological Data. Among the 107 patients studied, 98 (92%) were men and 9 (8%) women, with a mean age of 61.5 years (range, 38–75 years). All of the patients had Eastern Cooperative Oncology Group performance status of 0 or 1 and normal abdominal computed tomograms. Patients underwent lobectomy (87% of cases) or pneumectomy (13% of cases) with hylar and mediastinal lymph nodes sampling and in all of the cases were at stage I ($T_{1–2}N_0M_0$). Histological classification and grade were assessed by light microscopy according to WHO criteria (24). The most common histological type (53%) was the epidermoid carcinoma, followed by adenocarcinoma (32%), bronchioloalveolar carcinoma (9%), and anaplastic large cell carcinoma (6%). Thirthy-two (30%) tumors were well differentiated (G1), 39 (36%) moderately differentiated (G2), and 36 (34%) poorly differentiated (G3). Smoking history was available for 91 patients: 53 (58%) were smokers; 34 (38%) were former smokers (stopped smoking at least 1 year before the diagnosis of lung cancer); and 4 (4%) were nonsmokers.

Criteria for Evaluation of Telomerase Activity. The presence of a primer and a template for amplification in the TRAP-eze kit resulted in the formation of a 36-bp band in every lane (Fig. 1) and served as internal control to identify false negatives due to the presence of Taq polymerase inhibitors. A sample was considered positive for telomerase activity when the 36-bp internal control band and a ladder of PCR products similar to that of the telomerase-positive control lane were present (Fig. 1, *Lane*s *B*, *C*, *E*). Extracts that showed a 36-bp band but not ladders of PCR products were considered negative (Fig. 1, *Lane A*). The heat-inactivated samples (Fig. 1, *Lane D*) demonstrated a lack of the ladder pattern due to inactivation of the telomerase.

Telomerase Activity in NSCLCs Samples: Correlation with Pathological Parameters. Telomerase activity was detected in 66 (62%) of the 107 tumors examined (Fig. 2) and in none of the corresponding adjacent noncancerous lung tissue samples. Histologically, there were no obvious differences in the ratio of tumor cells to stromal cells in the tumors with and without detectable telomerase activity, and the reproducibility of the TRAP assay was confirmed by sampling multiple, different sites of some of the tumors. The distribution of telomerase activity was found to be dependent on the histopathological grade: low grade, G1 tumors were scored positive in 41% of cases, whereas G2 and of G3 tumors were positive in 72% and
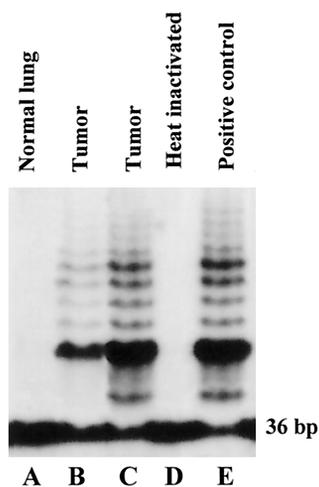
Fig. 1 Telomerase activity in NSCLC, normal lung tissue, and control samples. The 36-bp internal positive control band is seen in every lane. *Lane A*, telomerase-negative normal lung tissue extract showing only the 36-bp band. *Lanes B* and *C*, representative telomerase-positive NSCLC extracts showing the ladder pattern of PCR products, similar to that seen in *Lane E* (telomerase-positive control cell extract). *Lane D*, heat-inactivated telomerase-positive NSCLC extract.
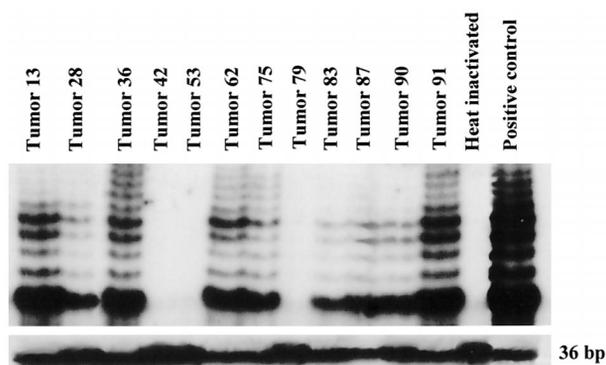

Fig. 2 Telomeric repeat amplification protocol analyses of NSCLC specimens are shown (*Tumors 13–91*). Tumors 42, 53, and 79 are examples of telomerase-negative lung carcinomas; the other tumor samples represent telomerase-positive cases. The *last two lanes* correspond to a heat-inactivated telomerase-positive NSCLC extract and to the telomerase-positive control cell extract.

Table 1 Comparison of tumor size, tumor histology, and histologic grade with telomerase activity in stage I NSCLC patients

Factor	Telomerase activity, n (%)		P
	Positive	Negative	
Tumor grade			
T_1	23 (68)	11 (32)	
T_2	43 (59)	30 (41)	NS[a]
Histology			
Squamous carcinoma	34 (60)	23 (40)	
Adenocarcinoma	24 (71)	10 (29)	
Bronchioloalveolar carcinoma	4 (40)	6 (60)	NS
Large cell carcinoma	4 (67)	2 (33)	
Histologic grade			
G1	13 (41)	19 (59)	
G2	28 (72)	11 (28)	0.0135
G3	25 (69)	11 (31)	

[a] NS, not significant.

Table 2 Statistical analysis of prognostic markers and clinical outcome in stage I NSCLC patients

Factor	Disease-free survival P	Overall survival P
Tumor size (T_1 *vs.* T_2)	0.1495 (NS)[a]	0.1375 (NS)
Histologic grade	0.8851 (NS)	0.9437 (NS)
Histotype	0.2574 (NS)	0.1723 (NS)
Telomerase activity	0.0115	0.0129

[a] NS, not significant.

69% of cases, respectively ($P = 0.0135$). A trend suggesting an association between telomerase activity and tumor histotypes was observed, but data were not statistically significant; 60% of squamous cell carcinomas, 71% of adenocarcinomas, 40% of bronchioloalveolar carcinomas, and 67% of large cell carcinomas were found to be positive for telomerase activity. Tumor size revealed no association with telomerase activity; T_1 tumors were positive in 68% of cases, and T_2 tumors were positive in 59% of cases. Results are summarized in Table 1. No correlation was observed between telomerase activity and age, sex, or smoking habits (data not shown).

Telomerase Activity and Clinical Outcome. The median follow-up in the series of patients examined was 54.5 months (range, 7–94 months). According to the Kaplan-Meier survival curves, the 5-year survival rate in the series of patients examined was 78%. Differences in survival of the patients were not significant in terms of T status, histotype, and histological grade of the tumor (Table 2). The median time to recurrence was 22 months (range, 3–56 months); the recurrence rate was 40 of 107 (37%); and the recurrences were initially located at a distant site in 30 cases (28%) or within the ipsilateral hemithorax in 10 cases (9%). There was no significant correlation between disease-free survival and T status, histological type, and histological grade of the tumor (Table 2).

Univariate survival curves (Fig. 3), estimated using the method of Kaplan and Meier, defined a significant association between telomerase activity and both disease-free survival ($P = 0.0115$) and overall survival ($P = 0.0129$; Table 2). It is remarkable that, in the 66 telomerase-positive patients, we observed 20 deaths, whereas only 3 deaths were seen in the 41 telomerase-negative cases. The joint effect of covariables that were significant at the 0.25 level in univariate analysis was examined using stepwise Cox regression. The histological grade was also included among the covariables in the Cox regression analysis because of the significant association observed between telomerase activity and grade. Therefore, factors included in the model were telomerase activity, tumor size, histological type of tumor, and histological grade. In multivariate analyses, the presence of telomerase activity remained the only strong predictor of disease-free survival ($P = 0.0173$; risk ratio = 1.682) and overall survival ($P = 0.0187$; risk ratio = 1.675).
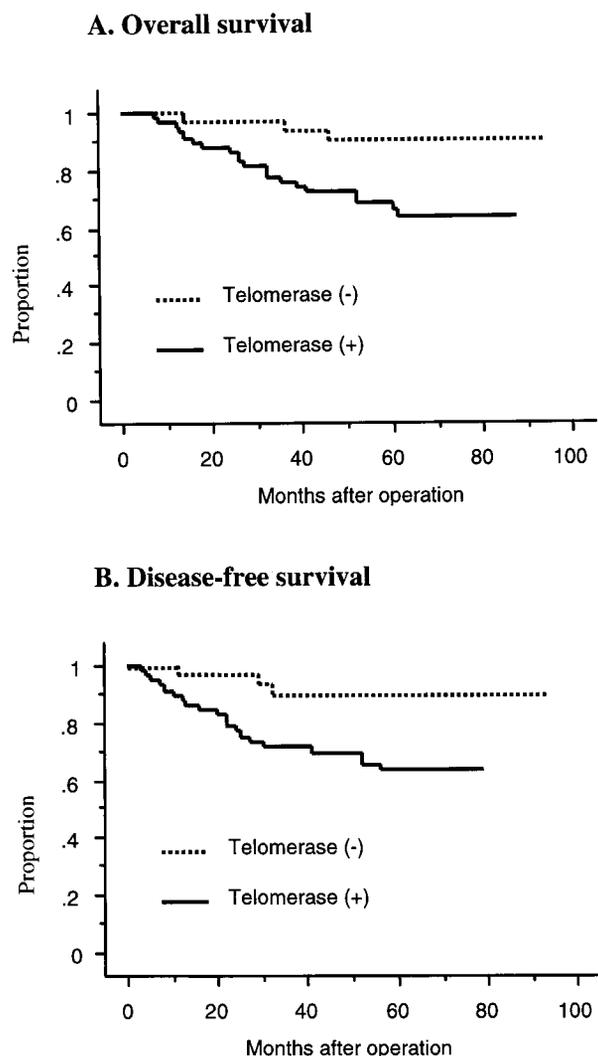
A. Overall survival

B. Disease-free survival

Fig. 3 Association between telomerase activity and overall survival (*A*) and disease-free survival (*B*).

DISCUSSION

Several biological parameters have been investigated as prognostic factors in patients with primary lung cancer, including oncogenes and tumor suppressor genes alterations, DNA ploidy, cell replication index, blood groups antigens, and neovascularization among others (25–27). Telomerase activity has been described as a marker of tumor aggressiveness and poor prognosis in different forms of human malignancies (12–17). In NSCLC patients, Hiyama *et al.* (19) observed high levels of telomerase activity in primary tumors and corresponding metastatic lesions. In some cases, the metastases were positive even when telomerase activity was undetected in the matching primary tumors, which suggests that activation of the telomerase enzyme may be involved in the metastatic process. The prognostic meaning of telomerase activity in NSCLC patients has recently been investigated by Albanell *et al.* (18). These authors studied 99 tumors obtained from consecutive operable NSCLC patients and found a weak trend toward an association between telomerase activity and unfavorable prognosis. Moreover, they observed that telomerase activity was not associated with clinical outcome in a multivariate Cox proportional hazards analysis adjusted for tumor stage and lymph node status. However, the number of stage I patients in the series studied by Albanell *et al.* was too low to draw definitive conclusions. On the basis of these results, we decided to evaluate the telomerase activity in a large series of NSCLC patients without metastatic spread. This project was based on a well-characterized series of consecutive operable patients with pathological stage I NSCLC from a single institution. These patients had standardized therapy (surgical resection only) and long-term complete follow-up, and did not have any confounding outcome variables, such us poor performance status, positive lymph nodes, or distant metastasis. One hundred seven stage I NSCLCs from as many patients were investigated by the TRAP assay to detect telomerase activity. We found high levels of the telomerase enzyme in 62% of these tumors. Telomerase activity was found to be a significant predictor of overall survival ($P = 0.0129$) in this panel of NSCLC patients. In addition, in the same series of patients, telomerase activity was a marker of disease-free survival ($P = 0.0115$). Other pathological variables evaluated, including tumor size, histological type, and histological grade of the tumors, were not significantly associated with diminished overall survival and disease-free survival in these patients. A Cox proportional hazards model that included telomerase activity and three other pathological variables (tumor size, tumor histology, and histological grade) still showed that the activity of the telomerase enzyme had a significant independent predictive power for both overall survival ($P = 0.0187$) and disease-free survival ($P = 0.0173$). Our data indicate that telomerase activity can be an important prognostic factor that should be validated in future prospective multi-institutional trials of adjuvant therapy for high-risk stage I NSCLC patients.

In the present series of stage I NSCLCs, telomerase activity was associated with histological tumor grade; the frequency with which the telomerase enzyme was activated in well-differentiated (G1) tumors was significantly lower than that observed in poorly differentiated (G2-G3) forms. This association, never observed before in NSCLCs, has been reported in other human malignancies such as brain tumors, cervical carcinomas, and tumors of the oral mucosa, but not in gastric, endometrial, or renal cancer (28–33).

In our series of tumors, a trend indicating an association between telomerase activity and tumor histotype has emerged, but, probably because of the low number of cases in some histological forms, data were not statistically significant. In particular, among the different histological types, adenocarcinomas showed the highest percentage (71%) of tumors with telomerase activity, whereas bronchioloalveolar carcinomas showed the lowest percentage (40%). This is particularly interesting because a debate is in progress about bronchioloalveolar carcinomas; some investigators think that they are not an entity distinguishable from adenocarcinomas. Even if limited to a low number of cases, our data suggest that telomerase activity could help in distinguishing these two histological forms. A larger series of adenocarcinomas and bronchioloalveolar tumors should be investigated to clarify this point. No association was observed between telomerase activity and tumor size; the fre-

quency of cases that scored positive for the telomerase enzyme was similar in T_1 and T_2 tumors.

In conclusion, our results indicate that telomerase activity evaluated by TRAP may serve to mark stage I NSCLC patients at risk for recurrent disease after complete surgical resection and should be considered in future prospective studies in selecting patients for adjuvant therapy.

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