Prediction of Survival in Stage I Lung Carcinoma Patients by Telomerase Function Evaluation

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SUMMARY: Telomerase activity and telomerase reverse transcriptase (hTERT) expression are elevated in human malignancies. We have investigated telomerase activity measured by the telomeric repeat amplification protocol (TRAP) assay and hTERT levels by real-time RT-PCR in stage I non-small-cell lung carcinomas. The purposes of our study included the comparison of these two techniques in the assessment of telomerase function and the evaluation of their prognostic significance. Telomerase activity and hTERT levels were determined in 90 stage I non-small-cell lung carcinoma patients, using TRAP assay and real-time RT-PCR, respectively. Variables were analyzed by the χ^2 and Fisher exact tests. Survival was analyzed by the Kaplan-Meier method. Multivariate analysis was performed with the Cox's proportional hazards model. Telomerase activity was elevated in 60 (67%) carcinomas. hTERT was elevated in 43 (48%) carcinomas. Only 21 (23%) tumors had low telomerase function by both TRAP and hTERT expression levels. Telomerase activity and hTERT were significantly correlated ($p = 0.017$), although 35 cases displayed discordant results. Both telomerase activity and hTERT levels were significantly associated with poor patient overall and disease-free survival ($p = 0.019$ and $p = 0.018$ for TRAP, and $p = 0.011$ and $p = 0.012$ for hTERT, respectively). Among the 21 patients with tumors displaying low telomerase function, defined by both TRAP and hTERT expression levels, only one succumbed to the disease ($p = 0.0053$). Our results suggest that the two techniques used in this study evaluate separate aspects of telomerase function and their combination provides powerful prognostic information in lung cancer patients. (*Lab Invest 2002, 82:729–736*).

*L*ung carcinoma is the first cause of death by cancer in developed countries, and its incidence is steadily increasing in the world (Greenlee et al, 2001). Patients' prognosis is largely dependent on the disease stage. The standard treatment regimen for patients with a traditionally better outcome, those grouped as stage I non-small-cell lung cancer (NSCLC), is resection of the primary tumor without adjuvant therapy. This treatment can cure about two-thirds of the patients with stage I disease, although the remaining 30% to 40% of the patients will have tumor recurrence and will die despite complete resection (Bunn et al, 1994; Strauss et al, 1995). Because careful pathologic examination of the primary lesions cannot be used to accurately predict clinical outcome, there exists a great need to identify molecular markers with which to define a subset of stage I NSCLC patients as candidates for new investigational adjuvant therapies, eventually leading to an improvement in patient survival.

Much interest has recently focused on telomerase, a ribonucleoprotein enzyme that uses its own integral RNA as a template for synthesis of telomeric repeats to compensate for the normal loss of terminal DNA sequences during cell divisions (Greider and Blackburn, 1985; Morin, 1989). After the introduction of the PCR-based assay, called the telomeric repeat amplification protocol (TRAP), telomerase activity was found to be present in most human tumors (Piatyszek et al, 1995; Shay and Wright, 1996). Furthermore, a correlation between telomerase activity and clinical outcome in different malignancies has been reported (Clark et al, 1997; Hiyama et al, 1995a, 1995b). We have previously shown that telomerase activity, evaluated by the TRAP assay, is an independent prognostic marker in stage I NSCLC patients (Marchetti et al, 1999).

The constituents of the telomerase complex have recently been identified, and human telomerase re-

verse transcriptase (hTERT) has been found to be responsible for the enzymatic activity of telomerase (Kilian et al, 1997; Nakamura et al, 1997; Nakayama et al, 1998). The expression of hTERT is thought to have important prognostic significance in different forms of human malignancies. In particular, hTERT mRNA overexpression evaluated by real-time PCR has been correlated with the histopathologic grade and receptor status in breast cancer, therefore identifying the tumors with more aggressive behavior (Bieche et al, 2000). An increase in hTERT mRNA expression was also correlated with the pathologic grade and clinical stage in bladder carcinomas, discriminating between low- and high-grade tumors and between superficial and muscle-invasive cancers (de Kok et al, 2000). A significant correlation between hTERT mRNA levels and telomerase activity was established in hepatocellular carcinomas (Hisatomi et al, 1999). Interestingly, these authors found a significant difference between tumor and non-neoplastic liver tissues, suggesting a possible application of hTERT quantification in the follow-up evaluation of patients at high risk to develop hepatocellular carcinomas. In prostate cancer, elevated hTERT levels were significant associated with myc overexpression, although both parameters were not correlated with tumor stage or Gleason grade (Latil et al, 2000).

In lung cancer, hTERT expression has been associated with telomerase activity, but no relationship with clinical and pathologic features has been reported (Arinaga et al, 2000). Recently, poor survival of lung cancer patients has been linked to high telomerase expression levels evaluated by in situ hybridization and immunohistochemistry, together with a semi-quantitative TRAP assay (Kumaki et al, 2001). A shorter survival was observed in patients with hTERT expression, evaluated by nonquantitative RT-PCR, indicating that hTERT expression might be an independent negative prognostic factor (Komiya et al, 2000). The prognostic significance of hTERT mRNA levels, assessed by current quantitative techniques, in lung carcinomas has yet to be elucidated.

In this study we report a comprehensive analysis of the telomerase status, based on the evaluation of hTERT mRNA expression and telomerase activity, in stage I NSCLC. A large series of stage I NSCLC patients was investigated for hTERT mRNA expression by real-time RT-PCR and for telomerase activity by the TRAP assay. The purposes of our investigation included: (a) the evaluation of real-time RT-PCR for the quantitative assessment of hTERT; (b) the comparison of hTERT expression and telomerase activity in lung tumors; and (c) the assessment of the prognostic utility of these two parameters, both individually and combined.

The results of our investigation suggest that these two techniques evaluate separate aspects of telomerase function. Their combination provides a better overall assessment of telomerase function and powerful prognostic information in lung cancer patients.

Results

hTERT Expression in Normal Lung and in Tumors

Ten normal lung tissue samples were used to establish a basal level of hTERT mRNA. All of the normal samples expressed detectable, but very low levels of hTERT mRNA, ranging from 0.01 n to 0.82 n.

Primary tumors from 90 stage I NSCLC patients were examined. Twenty-one (23%) cases showed very low levels of hTERT mRNA, comparable to the normal counterparts, whereas 69 (77%) tumors displayed hTERT levels higher than the normal samples. The range of hTERT levels varied greatly, ranging from 1 n to 939 n. Setting a cut off at 2.6 n, a value that represents the median value of expression distribution of the lung tumors, 47 (52%) cases had low hTERT expression and 43 (48%) cases high hTERT expression. hTERT levels, excluding the highest one, which greatly exceeded the range in tumor samples, are shown in Figure 1. The differences between normal lung hTERT levels and low and high hTERT expression tumors are statistically significant ($p = 0.00065$ and $p = 0.00001$).

Telomerase Activity

A sample was considered positive for telomerase activity when the 36-bp internal control band (see "Materials and Methods") and a ladder of PCR products similar to that of the telomerase-positive control lane were present. Extracts that showed a 36-bp band but not ladders of PCR products were considered negative (Fig. 2).

Telomerase activity was detected in 60 (67%) of the 90 tumors examined. There were no obvious differences in the ratio of tumor cells to stromal cells in the carcinomas 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.

Comparative Evaluation of Techniques with Pathologic Correlation

No correlation was observed between telomerase activity and/or hTERT expression and age, sex, smoking habits, tumor size, and tumor histotype. A trend suggesting an association between telomerase status and histopathologic grade of the tumors was observed, but data were not statistically significant. G1 tumors were scored positive in 20% of cases, whereas G2 and G3 tumors were positive in 42% and 38% of cases, respectively, $p = 0.11$. Results are detailed in Table 1. According to the telomerase activity results, 21 cases were devoid of telomerase activity and hTERT expression, whereas 69 showed activity and/or expression. Applying a χ^2 test, we observed a statistical link between hTERT expression and telomerase activity ($p = 0.017$), although 35 cases displayed discordant results (Table 2).
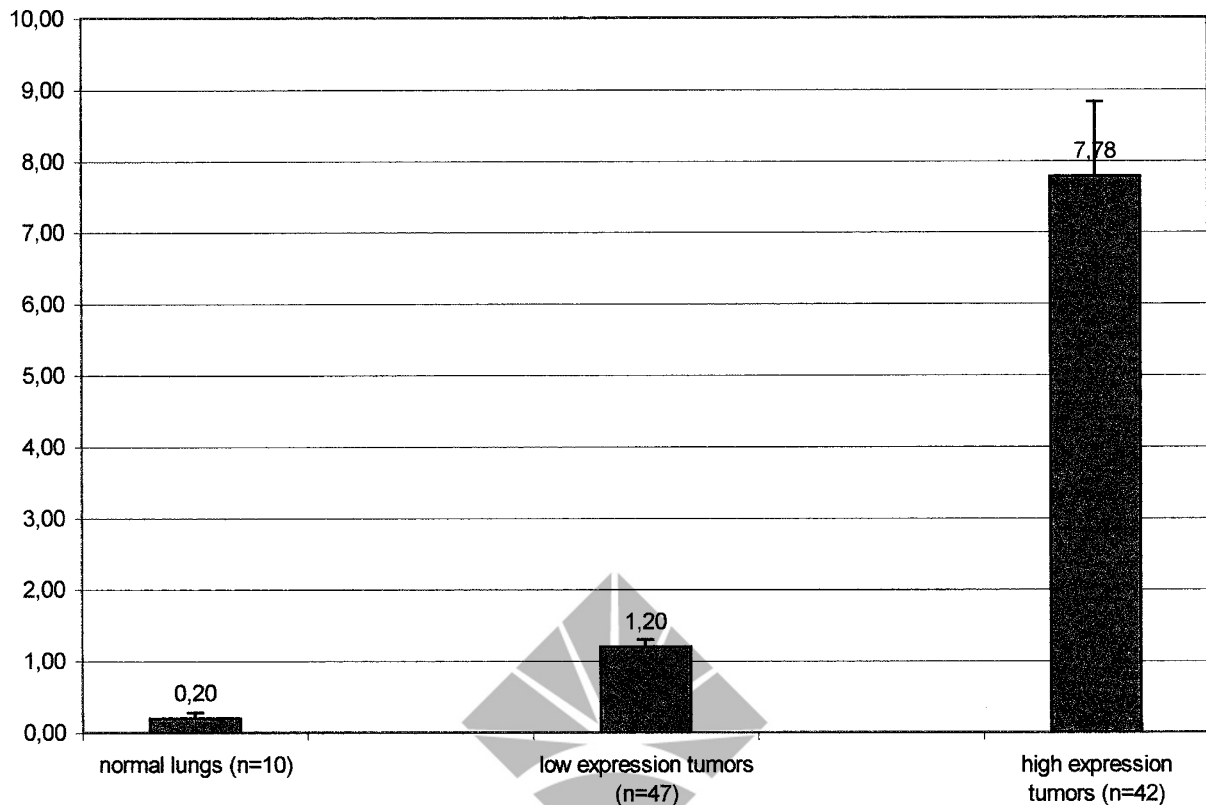
Figure 1.

Distribution of human telomerase reverse transcriptase (hTERT) expression levels in normal lung specimens, and in low- and high-expression tumors. Data are expressed as mean and standard error of the mean for each group. The tumor sample with extremely high expression level (939 n) has been excluded from the high-expression group.
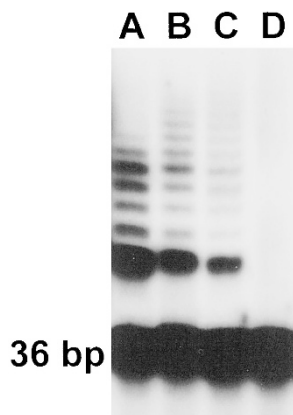
Figure 2.

Telomeric repeat amplification protocol analyses of non-small-cell lung cancer specimens are shown. Tumors A, B, and C are examples of telomerase-positive lung carcinomas, Tumor D represents a telomerase-negative case. The 36-bp internal positive control band is present in every lane.

Survival Analysis

The Kaplan-Meier survival curves in all patients with stage I NSCLC defined a significant association between telomerase activity and both disease-free ($p = 0.018$) and overall survival ($p = 0.019$). This correlation is preserved considering hTERT levels alone ($p = 0.012$ and $p = 0.011$, respectively). Among the 21 patients with tumors showing low telomerase function by both TRAP and hTERT expression levels, only one succumbed to the disease ($p = 0.0053$). Overall survival curves are shown in Figure 3.

A multivariate analysis, using Cox's proportional hazards regression model, was performed to investigate the independent prognostic factors among these markers: tumor stage, histology, histologic grade, and telomerase function. As a results, the presence of telomerase activity or hTERT levels were identified as the only two independent predictor factors of disease-free survival ($p = 0.0317$, risk ratio, 3.36 [1.11–10.15]; and $p = 0.0449$, risk ratio, 2.87 [0.91–8.76], respectively) and overall survival ($p = 0.0318$, risk ratio, 3.34 [1.09–10.08]; and $p = 0.0457$, risk ratio, 2.84 [0.90–8.69], respectively). A very strong association has been documented when considering both telomerase activity and hTERT expression and disease free survival ($p = 0.0092$, risk ratio, 9.01 [1.16–71.43]) and overall survival ($p = 0.0095$, risk ratio, 8.62 [1.12–66.67]; Table 3).

Discussion

In the present study we have carried out a comprehensive analysis of telomerase function in a series of 90 stage I NSCLC lung cancer patients. We evaluated hTERT mRNA levels by real-time RT-PCR analysis and telomerase activity by TRAP assay. The specific goals

Table 1. Comparison of Clinical and Pathological Variables with the Presence of Telomerase Activity and/or hTERT Expression in Stage I NSCLC Patients

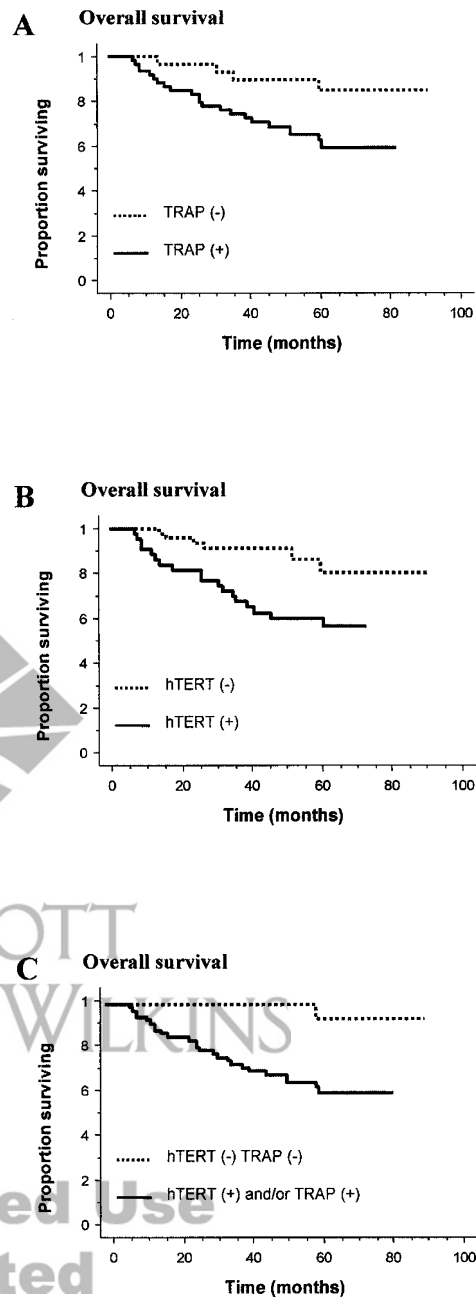| Factor	Telomerase activity and/or hTERT expression		
	Negative	Positive	*p* value
Age (mean ± SD, yr)	63.4 ± 7.2	63.1 ± 6.4	NS (0.7534)
Sex			
Male	19 (90%)	65 (94%)	NS (0.6209)
Female	2 (10%)	4 (6%)	
Smoking habits			
Smoker	13 (68%)	33 (57%)	
Former smoker	6 (32%)	24 (41%)	NS (0.6070)
Nonsmoker	0 (0%)	1 (2%)	
Tumor stage			
IA	6 (29%)	23 (33%)	
IB	15 (71%)	46 (67%)	NS (0.7932)
Histology			
Squamous carcinoma	12 (57%)	37 (54%)	NS (0.5288)
Adenocarcinoma	9 (43%)	28 (40%)	
Large cell carcinoma	0 (0%)	4 (6%)	
Histologic grade			
G1	9 (43%)	14 (20%)	
G2	7 (33%)	29 (42%)	NS (0.1101)
G3	5 (24%)	26 (38%)	

NS, not significant; NSCLC, non-small-cell lung cancer.

Table 2. hTERT Expression and Telomerase Activity in Stage I NSCLC

HTERT expression	Telomerase activity *n*/(%)		Total *n*/(%)	*p* value
	Positive	Negative		
High	34 (79)	9 (21)	43 (100)	0.017
Low	26 (55)	21 (45)	47 (100)	–

NSCLC, non-small-cell lung cancer.

Figure 3.

Overall survival curves according to the telomerase activity status by telomeric repeat amplification protocol (*TRAP*) assay (A), *hTERT* mRNA expression levels (B) and telomerase function, combining both telomerase activity and hTERT levels (C). All survival curve differences are statistically significant (see text for details).

of the study included the evaluation of real time RT-PCR for the quantitative assessment of hTERT, the comparison of the two techniques, and the assessment of their prognostic significance. We quantified the expression of the hTERT gene in 10 normal lung RNAs and 90 NSCLC RNAs. hTERT expression was present in all of the normal and tumor samples tested. However, all normal tissues and 21 (23%) lung tumors showed very low hTERT expression levels by real-time RT-PCR. When tumors were subdivided into two groups with low and high hTERT expression, according to the median value of expression distribution, a significant association was observed between high hTERT levels and shorter periods of disease-free and overall survival. To the best of our knowledge, this is the first report on the prognostic meaning of hTERT expression evaluated by real-time RT-PCR in NSCLC patients. Our data are in keeping with those recently reported using the same methodologic approach on a series of 134 breast cancer patients: high hTERT levels in primary tumors were found to be significantly associated with shorter relapse-free survival (Bieche et al, 2000). A correlation between real-time quantitative measurement of hTERT and clinicopathologic parameters of poor prognosis, such as histologic grade and muscle invasion, has also been observed in bladder urothelial cell carcinomas (de Kok et al, 2000).

Table 3. Statistical Analysis of Prognostic Markers and Clinical Outcome in Stage I NSCLC Patients

Factor	Disease-free survival		Overall survival	
	p	Risk ratio	*p*	Risk ratio
Tumor stage (IA, IB)	NS (0.2347)	1.70 (0.71–4.09)	NS (0.2204)	1.73 (0.72–4.17)
Histologic grade	NS (0.7455)	1.19 (0.42–3.38)	NS (0.8845)	1.08 (0.38–3.09)
Histotype	NS (0.5368)	1.27 (0.47–3.49)	NS (0.4339)	1.32 (0.49–3.68)
Telomerase activity and/or hTERT expression	0.0092	9.01 (1.16–71.43)	0.0095	8.62 (1.12–66.67)

NSCLC, non-small-cell lung cancer.

In this series of 90 NSCLC patients, telomerase activity, evaluated by an isotopic TRAP method, was present in 67% of cases and was found to be a predictor of clinical outcome, in keeping with our previous data (Marchetti et al, 1999). Because we found an expression of the hTERT gene in all of the samples, whereas telomerase activity was evident in 67% of the tumors, our data indicate that the sensitivity of the real-time RT-PCR method is much higher than that of the TRAP assay. The relationship between telomerase activity, detected by TRAP, and hTERT expression, investigated by real-time RT-PCR, in human tumors in vivo has been studied only in a limited number of cases. In 24 hepatocellular carcinoma samples Hisatomi et al (1999) found a significant correlation between the levels of hTERT mRNA and that of telomerase activity (r = 0.751). More recently, in agreement with our data, Buchler et al (2001) reported that in 29 pancreatic carcinomas the TRAP assay was positive in 35% of cases, whereas hTERT expression was present in 90% of cases. In our series of tumors, a significant association between high levels (above the median value) of hTERT expression and telomerase activity was observed, however, in 39% of cases results were discordant. These discrepancies may be explained by the following possibilities: (1) A high expression of hTERT in the absence of telomerase activity may be due to the presence of telomerase inhibitors in the tissue extract that may block the enzyme during the TRAP assay (Hohaus et al, 1996; Lancelin et al, 2000). (2) Low levels of hTERT gene expression in tumors with telomerase activity could be a consequence of the fact that hTERT mRNA and the catalytic subunit of the enzyme have different half-life periods.

We decided to evaluate the combined results obtained by TRAP and real-time RT-PCR: a stronger association between telomerase status and disease-free and overall survival has emerged. In particular, among the 21 patients who were scored negative with both methods, only 1 succumbed to the disease (*p* = 0.0053). Therefore, taken in a broad sense, telomerase alterations (overexpression and/or activity), which we call here telomerase function, are frequent events in NSCLC and strongly correlate with disease-free and overall survival. The results of the present study indicate that both techniques (real-time RT-PCR and TRAP assay) are indicated to provide the most accurate estimation of telomerase status in NSCLC. The use of a single technique could underestimate the

actual number of telomerase abnormalities in lung tumors. However, our data show that both methods used separately can give useful prognostic information.

The quantitative assessment of mRNA levels in human tissues by real-time RT-PCR may be hampered by the heterogeneity of samples, almost always containing both neoplastic and non-neoplastic cell populations. In our study we examined only samples in which tumor cells comprised at least 80% of the specimen, thus greatly reducing tissue heterogeneity. Indeed our results document hTERT levels much higher in tumor specimens than in non-neoplastic ones. In future investigations, for cases in which tissue heterogeneity is quantitatively more significant, the cell populations to be studied may be enriched using laser microdissection or flow cytometry and cell sorting techniques.

Real-time RT-PCR provides several advantages to quantify gene expression: (a) it does not require post-PCR sample handling, thereby avoiding problems related to carryover; (b) it has a high sample through-put and possesses a wide dynamic range, so that samples do not have to contain equal initial amounts of total RNA; (c) it makes RNA quantitation more precise and reproducible, because it is based on the threshold cycle values established in the early exponential phase of the PCR reaction (Gibson et al, 1996). At the present time, few papers have been published on hTERT expression evaluated by real-time RT-PCR in human tumors, and no data are available for lung cancer.

It is important to point out that the real-time RT-PCR technique has several advantages over the TRAP assay, in that it is faster, more reproducible, less sensitive to RNase activity, proteases, and protein inhibitors, and it can be applied to archival material for retrospective analyses (Specht et al, 2001). The TRAP assay may suffer some limitations primarily due to inefficient extraction, PCR inhibitors, telomerase inhibitors, and telomerase degradation in clinical samples. In addition, the TRAP assay provides only semi-quantitative data for telomerase activity evaluation (Wu et al, 2000).

In conclusion, using real-time quantitative RT-PCR, we have shown that the hTERT gene plays a major role in lung cancer progression. In particular, we found evidence that hTERT mRNA expression may serve as a new prognostic tool for stage I NSCLC patients. In addition, we demonstrated that telomerase activity is

significantly associated with hTERT expression and poor prognosis, but does not always reflect the status of hTERT mRNA. Finally, we brought evidence that a new parameter obtained by cumulating the results of the real-time RT-PCR and the TRAP assay is a strong indicator of clinical outcome. These new prognostic markers should be considered in future prospective trials to define high-risk stage I NSCLC patients who could benefit from adjuvant therapy.

Materials and Methods

Study Population

The primary NSCLC specimens were obtained from 90 patients with pathologic stage I NSCLC surgically treated at the Department of Surgery, University of Pisa (Pisa, Italy) between 1993 and 1994. 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 minutes of excision and stored at $-80°$ C. Immediately adjacent pieces of tumor and normal tissue were fixed and processed for light microscopy. In all tumor specimens the amount of tumor cells equaled or exceeded 80% of the overall sample, confirmed by histopathologic examination. Similarly, all of the macroscopically normal samples were judged to be benign.

The study population consisted of 84 (93%) men and 6 (7%) women, with a mean age of 63.3 years (range, 43 to 74 years). Patients underwent lobectomy (85% of cases) or pneumonectomy (15% of cases) with hylar and mediastinal lymph node sampling. Patient stage at the time of diagnosis was determined according to the guidelines of the American Joint Committee on Cancer (Fleming et al, 1997). Histologic type and tumor cell differentiation were determined according to the WHO criteria (Travis et al, 1999). The most common histologic type was squamous cell carcinoma (49 cases, 54%), followed by adenocarcinoma (37 cases, 41%) and large cell carcinoma (4 cases, 5%). Twenty-three (26%) tumors were well differentiated (G1), 36 (40%) moderately differentiated (G2), and 31 (34%) poorly differentiated (G3). Smoking history was available for 77 patients: 46 (60%) were smokers, 30 (39%) were former smokers (stopped smoking at least 1 year before the diagnosis of lung cancer), and 1 (1%) was a nonsmoker.

Follow-up data of the study population were obtained by direct patient contact. Follow-up occurred at 2-month intervals for the initial 2 years and at 4-month intervals afterward. Recurrences were detected by computed tomography scans or scintigrams and confirmed by pathologic examination, using biopsy specimens. 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 died of cancer-unrelated causes. Time in days from the date of the operation to the date of follow-up or death was recorded. The median follow-up in the series of patients examined was 54 months (range, 7 to 94 months).

TRAP Assay

Frozen tissue samples (50 to 100 mg) were homogenized with a pestle in 100 μl 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, 5 mM β-mercaptoethanol). After maintenance at $4°$ C for 30 minutes, the lysate was centrifuged at 12,000 $\times g$ for 20 minutes at $4°$ C. The supernatant was removed and its protein concentration was measured with the Biorad protein assay kit (Biorad Laboratories, Munchen, Germany). 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, Gaithersburg, Maryland; catalogue no. S7700). 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 and served as internal control to identify false negatives due to the presence of *Taq* polymerase inhibitors.

Real-Time RT-PCR

hTERT expression in these tumors was measured by real-time quantitative RT-PCR, based on TaqMan methodology, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, California). This technique allows, by means of fluorescence emission, to find the cycling point when PCR product is detectable (C_t value or threshold cycle). As previously reported, the C_t value correlates to the starting quantity of the target mRNA (Heid et al, 1994). To normalize the amount of total RNA present in each reaction, we amplified the housekeeping gene β-actin, which is assumed to be constant in both normal samples and lung tumors.

Our results are expressed as relative levels of hTERT mRNA, referred to a sample, called calibrator, chosen to represent 1X expression of this gene. The calibrator used was a lung carcinoma of the tissue collection under study, arbitrarily selected, that was analyzed on every assay plate with the unknown samples. All of the analyzed tumors express *n*-fold hTERT mRNA relative to the calibrator. Furthermore, one specific lung tumor sample was included in each run as control of reproducibility among experiments performed at different times.

The amount of target, normalized to an endogenous reference (β-actin) and relative to the calibrator is defined by the $\Delta\Delta C_t$ method as described by Livak K (Sequence Detector User Bulletin 2; Applied Biosystems). Specifically, the formula is applied as follows:

$$\text{target amount} = 2^{-\Delta\Delta C_t}$$

where $\Delta\Delta C_t = \{[C_t(\text{hTERT sample}) - C_t(\beta\text{-actin sample})] - [C_t(\text{hTERT calibrator}) - C_t(\beta\text{-actin calibrator})]\}$.

This method is based on the assumption that the target (hTERT) and β-actin display equal amplification efficiencies. To verify this condition, we checked ΔC_t (C_t hTERT $- C_t$ β-actin) variations according to tem-

plate dilution. To this end, we prepared a standard curve, composed of five different dilutions of MCF-7 total RNA, corresponding to 100, 25, 6.25, 1.6, and 0.4 ng. The slope of this curve was 0.049. To assure the appropriate amplification efficiency, the slope of the standard curve should be < 0.1.

RNA Extraction

Total RNA was extracted from frozen lung specimen by using a commercial kit, Trizol (Life Technologies, Gibco BRL), according to the manufacture's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through denaturing agarose gels. Only samples not degraded, that showed clear 18S and 28S bands under ultraviolet light, were used for real-time RT-PCR.

cDNA Synthesis

Total RNA (200 ng) was reverse-transcribed in a total volume of 50 μl containing 1X TaqMan buffer, 5.5 mM MgCl2, 1 mM deoxynucleotides, 2.5 μM random hexamers, 20 units RNase inhibitor, 62.5 units MuLv reverse transcriptase. The samples were incubated at 25° C for 10 minutes, 48° C for 30 minutes, and 95° C for 5 minutes.

PCR Amplification

PCR was performed in a total volume of 50 μl containing 1X TaqMan buffer, 5.5 mM MgCl2, 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 300 nM each primer, 100 nM probe, 0.5 units of AmpErase Uracil N-glycosilase (UNG), 1.25 units AmpliTaq Gold, and 10 μl of cDNA. Both β-actin and hTERT amplification were performed in duplicate for each sample.

The thermal cycling conditions included 2 minutes at 50° C and 10 minutes at 95° C, followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute. All reagents used for RT-PCR were purchased from Applied Biosystems.

Primers and Probes

Primers and probe for β-actin and hTERT mRNAs were chosen using a computer program, Primer Express (Applied Biosystems) The primers, placed in different exons, were tested not to amplify genomic DNA. Primers and probe nucleotide sequences for hTERT (GenBank accession number AF015950) were: forward primer 5'-CACGCGAAAACCTTCCTCA-3' (placed in exon 10; nt 2690–2708), reverse primer 5'-CAAGTTCACCACGCAGCC-3', TaqMan probe 5'(FAM)-CTCAGGGACACC-TCGGACCAGGGT-(TAMRA)3' (both placed in exon 11; nt 2755–2738 and nt 2734–2711, respectively).

Primers and probe for β-actin mRNA (GenBank accession number X00351) were: forward primer 5'-TCCTTCCT-GGGCATGGAG-3', reverse primer 5'- AGGAGGAGCAAT-GATCTTGATCTT-3', TaqMan probe 5'(FAM)-CCTGT-GGCATCCACGAAACTACCTTC-(TAMRA)3'. Probes were purchased from Applied Biosystems.

Statistical Analysis

The different variables of the tumors analyzed were tested for association using the χ^2 and Fisher exact tests. Overall survival was estimated by the method of Kaplan-Meier (Kaplan and Meier, 1958) and differences between curves were tested for statistical significance with the log-rank test (Cox and Oakes, 1984). Cox's proportional hazards regression models (Cox, 1972) were used to assess the independent prognostic contribution of clinicopathologic variables. The statistical analysis was performed using the Stat-View 5 statistical software run on a PowerPC G4 Macintosh computer.

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