Cooperative Role of Telomerase Activity and *p16* Expression in the Prognosis of Non–Small-Cell Lung Cancer

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Purpose: **Telomerase activity and *p16* expression can be considered two of the most important molecular markers implicated in tumorigenesis. Our main aim was to study the cooperative role of both molecular alterations in the prognosis of patients surgically resected for non–small-cell lung cancer (NSCLC).**

Patients and Methods: **We have determined telomerase activity and *p16* expression in a series of 98 prospectively collected NSCLC specimens obtained from patients who had undergone surgery without other treatment. Telomerase activity was investigated by a telomeric repeat amplification protocol enzyme-linked immunosorbent assay–based procedure, and *p16* expression was examined by Western blot. Associations with survival were evaluated.**

Results: **Positive results for telomerase activity were found in 82% of the cases, and this variable correlated with poor differentiation and recurrence of tumors. Lack of *p16* expression was observed in 61% of tumors, and a significant association with tumor recurrence was also observed. By univariate analysis, both negative telomerase activity and *p16*-positive expression were significantly correlated with a better prognosis. Moreover, statistics for equality of survival distributions for telomerase, adjusted for *p16*, indicated a positive interaction between both parameters. For telomerase-positive tumors, *p16* expression emerged as a significant independent protective variable, as indicated by Cox multivariate analysis (relative risk [RR], 0.214; *P* = .014). This protective effect was maintained only for stage I and II tumors (RR, 0.108; *P* = .046).**

Conclusion: **These results suggest that the combined telomerase activity and *p16* expression analyses may be of prognostic importance in NSCLC, especially for patients affected by stage I and II tumors.**

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THE EXPRESSION OF telomerase, the enzyme that synthesizes telomeric DNA de novo, is suppressed in most normal human somatic cells but is reactivated during tumorigenesis. This reactivation seems to arrest the normal loss of telomeric DNA incurred as somatic cells divide. Because continual loss of telomeric DNA is predicted to eventually limit cell proliferation, activation of telomerase in cancer cells may be an important step in the acquisition of cell immortalization, which occurs during tumor progression.[1,2] Recently, it has been shown that telomerase activity is not sufficient for immortalization of human keratinocytes or mammary epithelial cells. However, the downregulation of *p16* expression in combination with telomerase activity is able to immortalize epithelial cells efficiently.[3]

The loss of cell-cycle inhibition by negative regulators, such as *p16*, frequently occurs in certain primary malignant neoplasms, and *p16* is considered a major target in carcinogenesis. Its mechanism of action involves binding to and inactivating the D-cyclin-dependent kinase 4 (or 6) complex and, thus, renders the retinoblastoma protein inactive. This effect blocks the transcription of important cell-cycle regulatory proteins and results in cell-cycle arrest.[4-7] Lack of expression of *p16* has been associated with different abnormalities that occur in the gene, including homozygous deletion, methylation of the promoter, and point mutations.[7]

Non–small-cell lung cancer (NSCLC) represents one of the most frequent fatal malignancies in the world. Among men in Spain, lung cancer is the leading form of cancer, and squamous cell carcinoma (SCC) is the main histologic subtype. Spanish women have one of the lowest average incidences of the European cancer registers.[8,9] Although much is known about the causes, clinical features, and molecular pathogenesis of NSCLC, there is no molecular marker that has major clinical prognostic predictive value. Both telomerase activity and *p16* expression have been separately considered to be of importance in relationship to the development and to predict clinical outcome in NSCLC.

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Thus, it has been reported that telomerase activity may be useful both as a diagnostic marker to detect the existence of immortal lung cancer cells in clinical materials and as a target for therapeutic intervention.[10]

On the other hand, alterations in the molecular machinery that controls the transition from G_1 to S phase might represent central events that lead to NSCLC generation. To this respect, alterations in the *p16*/retinoblastoma (Rb) pathway have been considered in tumors from different origins,[7] including NSCLC.[11] According to these reports, *p16* abnormalities are a frequent event in this pathology, and data suggest that the reduction or loss of *p16* expression correlates with a worse patient outcome.[12]

Therefore, according to results published previously, telomerase activity and *p16* expression can be considered two of the most important molecular markers implicated in the development of NSCLC. However, there are no reports that examine abnormalities that affect both parameters in a cohort of patients affected by this pathology. In this context, our main aim was to study the prognostic value of telomerase activation in combination with *p16* expression in 98 patients surgically resected for NSCLC. Data reported here could expand the ability of clinicians to better predict clinical outcome and lead to better therapeutic strategies that can improve the clinical course of NSCLC.

PATIENTS AND METHODS

Patients and Tumor Samples

Ninety-eight non–small-cell lung tumors and their corresponding control tissue samples were obtained from patients who underwent radical surgery between 1994 and 1999 at San Carlos Hospital in Madrid. None of these patients had received neoadjuvant therapy before surgical resection. Informed consent was obtained from patients before investigation. Six patients were female, and 92 were male, with an average age of 63.85 ± 9.33 years (range, 40 to 83 years). The median follow-up period for patients was 37.6 months (range, 11 to 79 months). During surgery, tissue samples were obtained from tumoral specimens and from macroscopically normal lung parenchyma (at least 10 cm from the distal margin of the tumor, when possible). All tissue samples were snap-frozen in liquid nitrogen immediately after surgery and stored at −80°C. Cryostat-sectioned, hematoxylin-eosin–stained samples from each tumor block were examined microscopically by two independent pathologists to confirm the presence of more than 80% tumor cells. Paired normal tissues from the same patient, used as controls, were also microscopically confirmed. Cancers were staged pathologically using the tumor-node-metastasis system.[13] Forty-two patients had stage I tumors; eight had stage II; 37 had stage IIIA; nine had stage IIIB, and two patients had stage IV tumors. Therefore, 87 patients who had stage I, II, or IIIA tumors were subjected to curative surgery, whereas only a biopsy was taken from patients who suffered from more extensive disease. According to World Health Organization criteria, 56 tumors were SCC; 38 were adenocarcinomas (AC); and four were large-cell undifferentiated carcinomas. The histologic classification of tumors was established according to previous criteria.[14] Thus, 26 tumors were classified as well differentiated; 46, moderately; and 26, poorly differentiated.

Analysis of Telomerase Activity

Telomerase activity in tumor and normal tissues was analyzed, as previously published,[15] using a telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) method, which is an extension of the original telomeric repeat amplification protocol described by Kim et al.[16] Briefly, tissue samples were lysed in ice-cold lysis buffer for 30 minutes. In a first step, a volume of cell extract containing 6 μg of total proteins was incubated with a biotin-labeled synthetic telomerase-specific primer, and under established conditions, telomerase present in cellular extracts adds telomeric repeats (TTAGGG) to the 3' end of the primer. In a second step, these elongation products were amplified by PCR using specific primers. An aliquot of the PCR products was denatured, hybridized to a digoxigenin-labeled, telomeric repeat-specific probe, and bound to a streptavidin-coated microtiter plate. The immobilized PCR products were then detected with an antibody against digoxigenin that is conjugated to peroxidase. Finally, the probe was visualized by virtue of peroxidase-metabolizing TMB to form a colored reaction product and semiquantified photometrically (450 nm). Thus, considering that the cutoff for telomeric repeat amplification protocol–ELISA negativity corresponds to optical density $(OD)_{450nm}$ less than 0.2, we divided telomerase-positive cancers into three groups: tumors with high levels of telomerase activity ($OD_{450nm} \geq 2$; telomeric fragments added in the telomerase activity assay $>$ 86 bp); tumors with intermediate levels (OD_{450nm}, 1 to 1.999; telomeric fragments, 62 to 86 bp); and tumors that show low levels of telomerase activity (OD_{450nm}, 0.200 to 0.999; telomeric fragments $<$ 62 bp).

We used an extract of the telomerase-positive embryonic kidney cell line 293 as a positive control. The sensitivity of the procedure was sufficient to detect telomerase activity in an extract that contained 10 cells of the telomerase-positive cell line used as control. Negative controls were prepared in each case by treating cell extracts with DNase-free RNase. This treatment destroys telomerase activity because telomerase essentially requires the integrity of its internal RNA component as a template for the addition of the telomeric repeat sequences to the telomerase-specific primer.

Several authors have described occasional biopsies that contain inhibitors of Taq polymerase, and extracts that contain such inhibitors become amplifiable once the original extracts are diluted.[16-18] In our study, to avoid the effect of Taq polymerase inhibitors, we stimulated the activity of telomerase by serial dilutions of the extracts. Thus, for each of the samples, we evaluated telomerase activity directly in the cell extract (which contained 6 μg of total proteins) as well as in serial dilutions (1:10, 1:100, and 1:1000) established from the original extracts. Moreover, in all cases, we included a negative control. This protocol was applied both to tumor samples and the corresponding control tissues.

Alkaline Phosphatase Activity

Alkaline phosphatase activity was assayed as a control for possible protein degradation.[19] The enzyme activity was measured using a kinetic test in which *p*-nitrophenol is generated from *p*-nitrophenyl-phosphate as a result of the alkaline phosphatase activity. Our results indicated that all of the normal and tumor protein extracts considered in this study showed similar levels for this enzymatic activity (data not shown).

Evaluation of p16 *Expression by Western Blot*

Tissue samples of approximately 50 mg were quickly homogenized at 4°C in lysis buffer (0.5% NP-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 50 mmol/L Tris-HCl with pH of 7.5; 150 mmol/L NaCl with the following protease and phosphatase inhibitors: 20 mg/mL aprotinin, 10 mg/mL pepstatin, 1 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L sodium floride, and 1 mmol/L EDTA). The homogenates were centrifuged for 30 minutes at 13,000*g* at 4°C, and supernatants were collected. The total protein concentrations in the extracts were quantitated by the Bradford method. Fifty micrograms of the total protein extract were boiled twice in SDS gel-loading buffer (100 mmol/L Tris-Cl pH: 6.8; 200 mmol/L dithiothreitol; 4% SDS, 0.2% bromophenol blue and 20% glycerol) and separated by electrophoresis in a 15% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Protran BA Nitrocellulose Tranfer Membranes; Schleicher & Schuell, Einbeck, Germany) at 15 V for 30 minutes in semidry transfer buffer (48 mmol/L Tris, 39 mmol/L glycine, 20% methanol, 0.04% SDS). Membranes were then blocked with 5% milk in 0.1% Tween-20 triethanolamine-buffered saline (TBST) buffer (10 mmol/L Tris-HCl with pH of 7.4 and 150 mmol/L NaCl) and washed in TBST. Incubation with the primary monoclonal antibody (antip16 Ab-1, Oncogene; CN Biosciences, Inc, Darmstadt, Germany) was performed under the manufacturer's recommended conditions in 1% milk TBST. Membranes were incubated with a secondary antimouse antibody linked to horseradish peroxidase, whose presence bound to the membrane was detected by using the ECL system (Amersham Life Science Ltd, Buckinghamshire, England). Twenty micrograms of HeLa cells protein extracts were used for each gel as positive controls. Beta-actin expression data were used for protein normalization.

Clinical Correlations

Telomerase activity and *p16* expression were assessed for potential associations with a number of clinicopathologic parameters, including patient age and sex as well as stage, histology, and differentiation of tumors. Associations of categorical variables were evaluated using the χ^2 test. A P value of less than .05 was judged to be significant. Distributions of disease-free survival (DFS) were estimated with the Kaplan-Meier method, and comparisons were made with log-rank and Breslow statistics. Results were considered significant for P values less than .05. For the survival analysis, only patients who had undergone potentially curative surgery (patients with tumor-node-metastasis stages I to IIIA tumors) were considered. Also excluded were patients who died in the postoperative period. Univariate and multivariate analysis were performed using the Cox proportional hazards model to identify which independent factors jointly had a significant influence on survival. Adjusted-rate ratios were calculated. The existence of interactions and proportionality assumptions were evaluated. Variables that showed a P value less than .15 in the univariate analysis were selected for the multivariate analysis. The null hypothesis was rejected in each statistical test when $P < .05$. Analysis was performed using SPSS for Windows (version 10.0; SPSS, Inc, Chicago, IL).

RESULTS

This study attempted to determine the effect of telomerase activity and *p16* expression on the prognosis of patients who had undergone potentially curative surgery for NSCLC. Telomerase activity was measured in tumor and nontumor tissues using a telomerase semiquantitative PCR-ELISA assay, the results of which correlated with the appearance of a telomerase-mediated 6 nucleotide-ladder, as we have previously published.[15] Thus, telomerase activity was investigated in 85 tumors because, in 13 cases, a partial RNA degradation was observed previous to the activity assay. Because RNA integrity is an essential parameter for telomerase activity evaluation, these samples were excluded. Thus, telomerase-positive tumors were classified into three groups according to activity levels: low, intermediate, and high activity. Twenty cases (23.5%) showed low activity levels, and intermediate or high levels were detected in 23 and 27 tumors (27.1% and 31.8%), respectively. In 15 cases (17.6%), negative telomerase values were obtained. Therefore, positive results for enzyme activity were found in 70 (82%) of the 85 NSCLCs. Moreover, weak levels of telomerase activity were found in 21 (24.7%) of the 85 normal tissues analyzed.

Our data indicated that poor differentiation of tumors was significantly associated with positive telomerase activity ($P = .015$). In addition, telomerase activity showed a borderline correlation with recurrence of tumors ($P = .071$). This variable did not show an association with other clinicopathologic features included in this study (Table 1). Tumor recurrence and telomerase activity were clearly associated in tumors staged within the groups I to IIIA that corresponded to patients who had undergone potentially curative surgery and who did not die in the postoperative period ($n = 69$; $P = .017$). In this case, we have found tumor recurrence in 21 (36.2%) of the 58 tumors that showed telomerase activity (eight tumors with low levels, two patients with intermediate activity, and 11 tumors with high enzyme activity). No cases of tumor recurrence were observed in the 11 telomerase-negative tumors. For tumors staged as I to IIIA, a significant correlation was also found between poor differentiation of tumors and positive telomerase activity ($P = .007$), with no differences between different activity levels.

p16 expression was estimated by densitometry of immunoblots (Fig 1). After beta-actin normalization, all nontumor tissues showed low or undetectable levels of protein expression (range, 0 to 0.26 arbitrary densitometric units), whereas tumor tissues expressed *p16* at different levels. Thus, tumors were divided into three groups according to protein expression in relation to *p16* levels in normal tissues: tumors with low, intermediate, and high *p16* expression. Low expression was defined as patients with *p16* levels similar to or lower than those in the corresponding control tissues. The group of intermediate-*p16* expression was established for tumors that overexpressed *p16* from 1.1-fold to three-fold in relationship to control tissues. Finally, tumor

Table 1. Telomerase Activity in Relation to Clinicopathology

	No. of Patients	Telomerase Activity								
		Low		Intermediate		High		Negative		
		No.	%	No.	%	No.	%	No.	%	*P*
Age, years	85	62.85 ± 6.69		65.78 ± 10.89		62.18 ± 9.71		65.85 ± 11.46		.423
Sex										
Male	79	20	25.3	22	27.9	25	31.6	12	15.2	.132
Female	6	0	0	1	16.7	2	33.3	3	50	
Stage										
I	38	11	28.9	8	21.1	10	26.3	9	23.7	.828
II	8	1	12.5	3	37.5	3	37.5	1	12.5	
IIIA	29	6	20.7	8	27.6	12	41.4	3	10.3	
IIIB	8	1	12.5	3	37.5	2	25	2	25	
IV	2	1	50	1	50	0	0	0	0	
Histology										
Squamous cell carcinomas	50	14	28	13	26	14	28	9	18	.305
Adenocarcinomas	32	6	18.8	8	25	13	40.6	5	15.6	
Large-cell undifferentiated carcinomas	3	0	0	2	66.7	0	0	1	33.3	
Differentiation										
Well	22	1	4.5	6	27.3	7	31.8	8	36.4	.015
Moderate	41	13	31.7	10	24.4	11	26.8	7	17.1	
Poor	22	6	27.3	7	31.8	9	40.9	0	0	
Recurrence										
Positive	25	9	36	5	20	10	40	1	4	.071
Negative	60	11	18.3	18	30	17	28.3	14	23.3	

samples with more than threefold levels of *p16* with respect to normal tissues were included in the high-expression group. According to our data, 60 tumor samples (61.2%) showed low *p16* levels, 17 (17.3%) showed intermediate levels, and a distinctly higher expression was found in 21 samples (21.4%). All correlations included here have been performed on the basis of *p16*-negative expression for tumors of the first group and *p16*-positive expression for tumors with intermediate or high *p16* levels. Thus, the percentage of tumors with positive expression of *p16* was 38.7% (38 of 98).

Expression of *p16* was not associated with age or sex of patients nor with stage, histology, or differentiation of tumors. However, a significant correlation was detected between lack of *p16* expression and tumor recurrence ($P = .030$) (Table 2). When we considered the group of tumors included in I to IIIA stage that corresponded to patients who survived into the postoperative period (n = 72), we detected a strong association between tumor recurrence and lack of *p16* expression ($P = .001$). In fact, only three of the 22 patients who experienced recurrence showed significant *p16* expression, whereas in 29 (58%) of 50 cases without recurrence, intermediate or high *p16* expression levels were detected. *p16*-positive expression was significantly correlated with intermediate or high telomerase activity levels (Table 3).

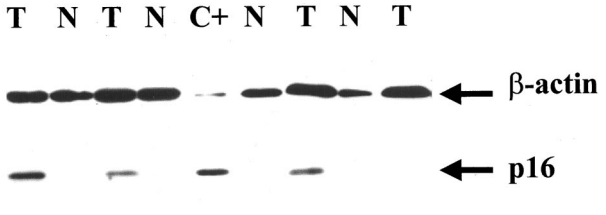

Fig 1. Examples of *p16* expression analysis by Western blot. N, normal tissues; T, tumor tissues; C+, positive control.

Next, we calculated the DFS from the date of surgery until the time of tumor recurrence. Analyses of these survival data only included patients who underwent curative surgery. In relation to telomerase activity, a significant difference on survival was found between negative and positive cases (log-rank $P = .04$). The 3-year survival probability was 100% in patients with telomerase-negative tumors and 57.7% in the group of patients who showed telomerase-positive tumors because 21 cases of tumor recurrence were detected within the 58 patients included in this group (Fig 2). When we considered different telomerase-positive levels, no significant differences in the relationship to survival probability were detected.

The lack of *p16* expression was also correlated with an adverse outcome in patients affected by NSCLC (log-rank $P = .004$). The 3-year survival probability was 46% in

Table 2. *p16* Expression in Relation to Clinicopathology

		p16 Expression				
		Negative		Positive		
	No. of Patients	No.	%	No.	%	*P*
Age, years	98	64.33 ± 9.30		63.1 ± 9.44		.528
Sex						
Males	92	56	60.9	36	39.1	.570
Females	6	4	66.7	2	33.3	
Stage						
I	42	24	57.1	18	42.9	.061
II	8	7	87.5	1	12.5	
IIIA	37	21	56.8	16	43.2	
IIIB	9	8	88.9	1	11.1	
IV	2	0	0	2	100	
Histology						
Squamous cell carcinomas	56	38	67.9	18	32.1	.185
Adenocarcinomas	38	19	50	19	50	
Large-cell undifferentiated carcinomas	4	3	75	1	25	
Differentiation						
Well	26	12	46.2	14	53.8	.306
Moderate	46	32	69.6	14	30.4	
Poor	26	16	61.5	10	38.5	
Recurrence						
Positive	30	23	76.7	7	23.3	.030
Negative	68	37	54.4	31	45.6	

patients without *p16* expression, compared with 88.2% in the group of patients with a significant value for *p16* expression. In fact in the first group, 29 of the 50 patients showed tumor recurrence, whereas only three recurrences within the 22 cases were detected in the second group (Fig 3).

By univariate analysis, stage IIIA tumors were associated with a significant survival reduction. Moreover, both negative telomerase activity and *p16*-positive expression were correlated with a better prognosis for patients (Table 4). Relative risk (RR) was significantly lower in patients whose tumors did not show telomerase expression (RR, 0.036; $P = .009$) or positive *p16* expression (RR, 0.205; $P = .003$).

On the other hand, statistics for equality of survival distributions for telomerase adjusted for *p16* had significant results with the Breslow test ($P = .03$). For negative *p16* expression, survival differences were significant ($P = .04$), whereas when *p16* expression was considered positive, we did not find significant differences between the two survival curves ($P = .65$) (Fig 4). These results indicated a positive interaction between the two parameters evaluated in this study, telomerase activity and *p16* expression.

To identify which independent factors jointly had a significant influence on survival, Cox multivariate analysis was performed. For this study, variables with a *P* value lower than .15 in the univariate analysis were selected. However, it was not mathematically possible to establish the Cox multivariate study, including telomerase activity, because one subset of events was empty (no cases of recurrence within the group of telomerase-negative tumors), and we would have obtained an undefined value for RR. So, we performed multivariate analysis only with telomerase-positive cases. Thus, our data indicated that, for patients affected by I to IIIA tumors who showed telomerase activity, *p16*

Table 3. Relationship Between *p16* Expression and Different Levels of Telomerase Activity

	Telomerase Activity									
	Negative		Low		Intermediate		High			
	No.	%	No.	%	No.	%	No.	%	Total	*P*
p16 expression										.026
Positive	11	21.1	14	26.9	8	15.3	19	36.5	52	
Negative	4	12.1	6	18.2	15	45.5	8	24.2	33	
Total	15		20		23		27		85	

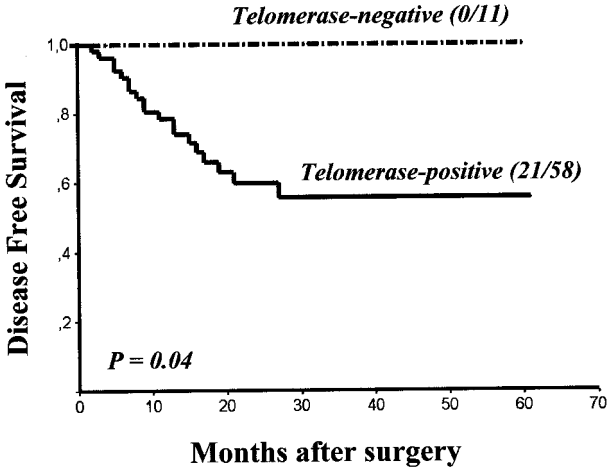

Fig 2. Kaplan-Meier survival curves show the association of telomerase activity with DFS.

expression was a significant protective variable independent of tumor stage. RR was approximately four times lower than in the case of tumors with negative *p16* expression (RR, 0.214; $P = .014$) (Table 5). Interestingly, the protective effect of *p16* expression was maintained when we only considered the earlier-stage tumors (stage I and II tumors). *p16*-positive expression can also be considered a protective biomarker for stage I and II tumors with positive telomerase activity (RR = 0.108; $P = .046$) (Table 5). In fact, Fig 5 shows the correlation between telomerase-positive activity and *p16* expression of tumors in association with patient prognosis, including stage I to IIIA tumors versus only including stage I and II tumors. In both cases, a high rate of relapse was detected in patients who had tumors that lacked *p16* expression ($P = .001$ and $P = .015$, respectively).

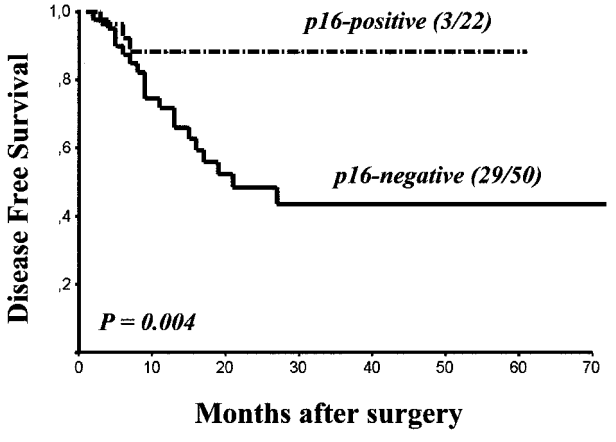

Fig 3. Relationship between *p16* expression status and DFS.

Table 4. Cox Univariate Analysis for Tumor Stage, Differentiation, Telomerase Activity, and *p16* Expression

	RR	95% Confidence Interval	*P*
Tumor stage			
II *v* I	1.887	0.500-7.121	.349
IIIA *v* I	2.525	1.013-6.292	.047
Differentiation			
Moderate *v* well	1.351	0.415-4.390	.617
Poor *v* well	2.001	0.560-7.146	.285
Telomerase, negative activity	0.036	0.000-6.516	.009
p16, positive expression	0.205	0.060-0.696	.003

DISCUSSION

Previous studies have demonstrated that detection of telomerase in human tissue samples may be one of the best markers of neoplasia across a broad range of tumor types.[20] Although the expression of telomerase does not by itself lead to a tumorigenic phenotype,[21] telomere maintenance might cooperate with additional oncogenic mutations to create a malignantly transformed clone.[22] Thus, it seems that, in certain primary cell types, senescence can only be bypassed by concomitant activation of telomerase and loss of either pRb or *p53* function.[23] These results indicated that the Rb pathway, along with a telomere maintenance pathway, plays a role in determining the life span of epithelial cells. Moreover, recent results in human epithelial cell lines indicate that immortalization may require both activation of telomerase and other genetic and/or epigenetic alterations that abrogate normal differentiation in $p16^{INK4a}$, $p14^{ARF}$, or other genes to become immortal.[24]

Despite the fact that a great number of studies regarding molecular abnormalities in relationship to cancer have emerged in recent years, there has been no report to specifically investigate telomerase activity in combination with *p16* expression in human cancer. So, considering the role of telomerase activity in cellular immortality and that *p16* is a major target in carcinogenesis, for which an involvement in cell senescence has been described,[7] we have evaluated both parameters in a cohort of NSCLC patients who only underwent surgical resection in this study.

Our data indicated that a high proportion of lung tumors (82%) showed positive telomerase activity, which correlated this marker with negative determinants of prognosis, such as poor differentiation or recurrence of tumors. These results were in agreement with previously published studies.[25] Moreover, in other reports, high telomerase activity had been frequently detected in primary NSCLC that exhibited high tumor-cell proliferation rates and advanced pathologic stage.[19] In contrast to these data and according to our results, no differences regarding clinicopathology of

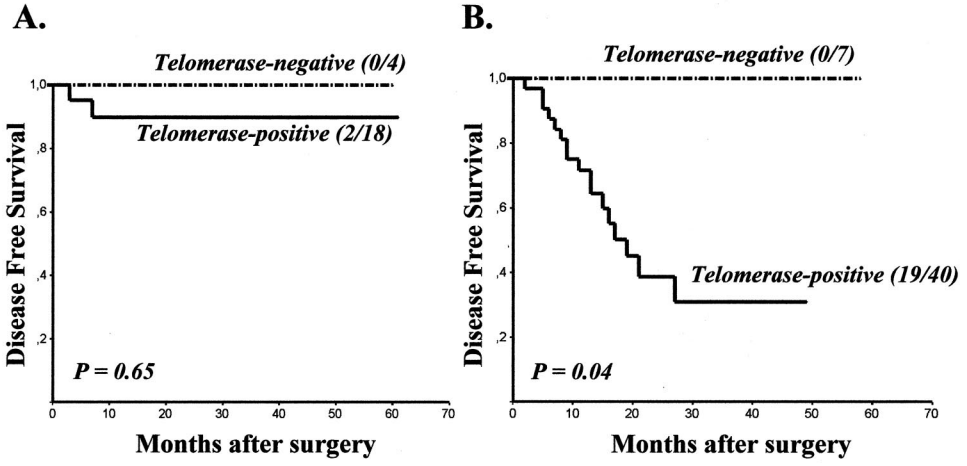

Fig 4. Kaplan-Meier survival curves show the association of telomerase activity and DFS in patients (A) affected by *p16*-positive tumors and (B) with *p16*-negative tumors.

tumors were detected between the distinct telomerase positive levels. In normal tissues, we detected weak levels of telomerase activity in 24.7% of cases. These data could be attributed to the presence of microscopic telomerase-positive tumor cells or the presence of connective and/or lymphocytic cells that may show positive telomerase activity levels.

In relation to *p16*, we found low or undetectable levels of protein expression in all of the control tissues considered and different levels of *p16* expression in tumor samples. Taking into account that, in most cases, our nontumor samples were collected at least 10 cm away from the distal margin of the tumors and, therefore, no contamination by tumor cells would be expected, these results may indicate that *p16* in tumor samples is upregulated in response to oncogenic factors. In fact, it has been suggested that upregulation of *p16* is not a consequence of the entry into senescence but is directly linked to the accumulation of cell doublings.[26] Moreover, in tumor samples, we found altered expression to be a rather frequent event in NSCLC because our results revealed a lack or low levels of protein expression in 61% of cases. Inactivation of the *p16* protein was demonstrated in 30% to 50% of NSCLC and in 20% to 40% of lung ACs, with the rate of *p16* inactivation lower in AC than that in SCC (40% to 75%).[27,28] Our present correlation analyses demonstrated that loss of *p16* expression was significantly correlated with tumor recurrence, but no other associations were observed with clinicopathologic features of tumors. These data are in agreement with the findings of Groeger et al,[12] who reported a high frequency of aberrant *p16* expression in NSCLC but did not correlate this finding with histopathologic parameters. Kratzke et al,[29] however, reported a high frequency of *p16* aberrant expression in NSCLC to be inversely related to the pathologic stage of the disease.

Table 5. Cox Multivariate Analysis for Tumor Stage and *p16* Expression in Telomerase-Positive Tumors

	RR	95% Confidence Interval	*P*
Stage I to IIIA tumors			
Tumor stage			
II *v* I	1.423	0.346-5.857	.625
IIIA *v* I	2.062	0.703-6.048	.187
p16, positive expression	0.214	0.062-0.733	.014
Stage I and II tumors			
Tumor stage			
II *v* I	1.353	0.325-5.625	.678
p16, positive expression	0.108	0.012-0.960	.046

Looking for correlations between telomerase activity and *p16* expression in lung carcinomas, we observed that tumors that showed moderate or high levels of telomerase activity were those in the groups with a higher proportion of cases that expressed *p16*. These results could indicate a possible mechanism to arrest cycling in cells with an extra capacity for proliferation that may result from telomerase reactivation in an earlier stage.

Next, we evaluated both telomerase activity and *p16* expression in relation to prognosis through DFS data. Very few studies have addressed the relationship between telomerase activity and prognosis in lung cancer, and controversial results have been obtained. Thus, although telomerase activity has been considered one of the most important prognostic factors in patients affected by NSCLC,[25] other authors have not found such association.[19] According to our

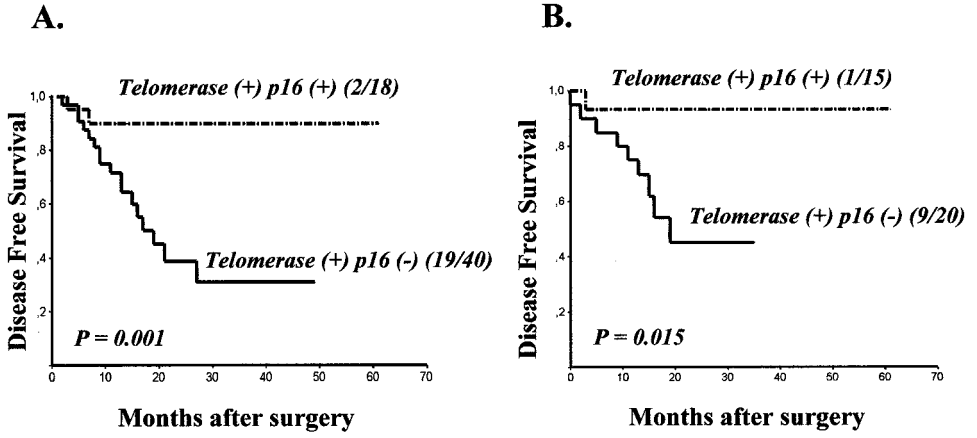

Fig 5. Correlation between *p16* expression status and DFS in telomerase-positive cases: (A) patients with stage I to IIIA tumors and (B) only patients with stage I or II tumors.

results, telomerase activity has emerged as a prognostic marker of importance in NSCLC. In addition, altered *p16* expression is an unfavorable prognostic factor for this pathology. Loss of *p16* expression correlates with a worse patient outcome. Previous studies examined *p16* expression by immunohistochemical techniques and revealed similar data.[12] Moreover, taking into account the high proportion of tumors with telomerase activity, we grouped telomerase-positive tumors by function of *p16* expression. Thus, our results revealed a positive interaction between these parameters, with *p16* expression as a protective variable independent of tumor stage, in patients who had developed tumors that showed positive telomerase activity. Our observations clearly provided in vivo evidence that supports recent proposals that multiple clocks function to limit the proliferative capacity of human cells. Thus, in human keratinocytes that express human telomerase reverse transcriptase, replicative potential was limited by a *p16*-dependent mechanism. Abrogation of this mechanism together with telomerase expression immortalizes keratinocytes without affecting other major growth control or differentiation systems.[30] It is possible that, in NSCLC cells that show telomerase reactivation, *p16* is upregulated to control cellular senescence under a mechanism sensitive to telomere length. Loss of this mechanism by the altered regulation of *p16* expression could provide an advantage to gain proliferation, with important prognostic implications in patients affected by NSCLC.

Therefore, considering that it is now highly likely that telomere maintenance contributes to oncogenesis, *p16* expression could reverse immortality in cells that have reactivated telomerase. Alternatively, the lack of *p16* expression could contribute to tumor progression and malignancy. These results in NSCLC and, more specifically, in the earlier stages of tumor progression, could be useful for the selection of patients with potentially unfavorable outcomes, to establish adjuvant therapy protocols.

REFERENCES

1. Counter CM, Meyerson M, Eaton EN, et al: Telomerase activity is restored in human cells by ectopic expression of hTERT catalytic subunit of telomerase. Oncogene 16:1217-1222, 1998

2. Meyerson M: Role of telomerase in normal and cancer cells. J Clin Oncol 18:2626-2634, 2000

3. Kiyono T, Foster SA, Koop JI, et al: Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396:84-88, 1998

4. Serrano M, Hannon GJ, Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366:704-707, 1993

5. Kamb A, Gruis NA, Weaver-Feldhaus J, et al: A cell cycle regulator potentially involved in genesis of many tumor types. Science 264:436-440, 1994

6. Lukas J, Parry D, Aagaard L, et al: Retinoblastoma protein-dependent cell cycle inhibition by the tumor suppressor *p16*. Nature 375:503-506, 1995

7. Liggett WH, Sidransky D: Role of the *p16* tumor suppressor gene in cancer. J Clin Oncol 16:1197-1206, 1998

8. Takkouche B, Gestal-Otero JJ: The epidemiology of lung cancer: Review of the risk factors and Spanish data. Eur J Epidemiol 12:341-349, 1996

9. Levi F, Lucchini F, Negri E, et al: Cancer mortality in Europe, 1990-1994, and an overview of trends from 1955 to 1994. Eur J Cancer 35:1477-1516, 1999

10. Hiyama K, Hiyama E, Ishioka S, et al: Telomerase activity in small-cell and non–small-cell lung cancers. J Natl Cancer Inst 87:895-902, 1995

11. Fong KM, Sekido Y, Minna JD: Molecular pathogenesis of lung cancer. J Thorac Cardiovasc Surg 118:1136-1152, 1999

12. Groeger AM, Caputi M, Esposito V, et al: Independent prognostic role of *p16* expression in lung cancer. J Thorac Cardiovasc Surg 118:529-535, 1999

13. Mountain CF: A new international staging system for lung cancer. Chest 89:225-233, 1986 (suppl)

14. Sobin L: The World Heath Organization's histological classification of lung tumors: A comparison of the first and second editions. Cancer Detect Prev 5:391-406, 1982

15. González-Quevedo R, de Juan C, Massa MJ, et al: Detection of telomerase activity in human carcinomas using a TRAP-ELISA method: Correlation with hTR and hTERT expression. Int J Oncol 16:623-628, 2000

16. Kim NW, Piatyszek MA, Prowse KR, et al: Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015, 1994

17. Wright WE, Shay JW, Piatyszek MA: Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. Nucleic Acids Res 23:3794-3795, 1995

18. Kim NW, Wu F: Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res 25:2595-2597, 1997

19. Albanell J, Lonardo F, Rusch V, et al: High telomerase activity in primary lung cancers: Association with increased cell proliferation rates and advanced pathologic stage. J Nat Cancer Inst 89:1609-1615, 1997

20. Urquidi V, Tarin D, Goodison S: Role of telomerase in cell senescence and oncogenesis. Annu Rev Med 51:65-79, 2000

21. Morales CP, Holt SE, Ouellette M, et al: Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat Genet 21:115-118, 1999

22. Hahn WC, Counter CM, Lundberg AS, et al: Creation of human tumor cells with defined genetic elements. Nature 400:464-468, 1999

23. Kiyono T, Foster SA, Koop JI, et al: Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396:84-88, 1998

24. Farwell DG, Shera KA, Koop JI, et al: Genetic and epigenetic changes in human epithelial cells immortalized by telomerase. Am J Pathol 156:1537-1547, 2000

25. Taga S, Osaki T, Ohgami A, et al: Prognostic impact of telomerase activity in non–small-cell lung cancers. Ann Surg 230:715-720, 1999

26. Serrano M: The tumor suppressor protein $p16^{INK4a}$. Exp Cell Res 237:7-13, 1997

27. Taga S, Osaki T, Ohgami A, et al: Prognostic value of the immunochemical detection of p16INK4 expression in non–small-cell lung carcinoma. Cancer 80:389-395, 1997

28. Kawabuchi B, Moriyama S, Hironaka M, et al: *p16* inactivation in small-sized lung adenocarcinoma: Its association with poor prognosis. Int J Cancer 84:49-53, 1999

29. Kratzke RA, Todd MG, Jeffrey BR, et al: Rb and p16INK4a expression in resected non–small-cell lung tumors. Cancer Res 56: 3415-3420, 1996

30. Dickson MA, Hahn WC, Ino Y, et al: Human keratinocytes that express hTERT and also bypass a *p16*(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol 20:1436-1447, 2000