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The Expression of Telomerase Reverse Transcriptase Protein is an Independent Prognostic Marker in Early Stage Non-Small Cell Lung Carcinomas

**Ji Han Jung · Chan-Kwon Jung
Ahwon Lee · Gyeongsin Park
Jinyoung Yoo · Kyo Young Lee**

Department of Hospital Pathology, College of Medicine, The Catholic University of Korea, Seoul, Korea

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Corresponding Author
Jinyoung Yoo, M.D.
Department of Hospital Pathology, St. Vincent's Hospital, The Catholic University of Korea, 93 Ji-dong, Paldal-gu, Suwon 442-723, Korea
Tel: 031-249-7593
Fax: 031-244-6786
E-mail: jinyyoo@vincent.cuk.ac.kr

Background : The catalytic subunit of telomerase, hTERT (telomerase reverse transcriptase), is one of the most important components of telomerase, and performs a pivotal role in the mechanism underlying the regulation of telomerase activity in cellular immortalization and carcinogenesis. The principal objective of this study was to investigate hTERT expression in patients with non-small cell lung carcinomas (NSCLCs), and to evaluate its clinical significance and association with the expression of p16 and p53. **Methods :** Using tissue microarray, the protein expression profiles of hTERT, p16 and p53 were investigated via immunohistochemistry in 167 samples of NSCLCs. **Results :** Expression was observed in 54.5% (91/167) of the tumors, which were predominantly squamous cell carcinomas. Patients evidencing hTERT expression in their tumors exhibited significantly poorer survival rates than did patients without hTERT expression in early-stage NSCLCs (p=0.0125). According to the results of our Cox regression analysis, hTERT expression proved to be an independent prognostic factor (p=0.006), particularly for squamous cell carcinomas (p=0.019). hTERT expression was not correlated with p16 expression, but was rather associated with the expression of p53 (p=0.002). **Conclusions :** Our results show that hTERT may perform a function in the progression of NSCLC, and that its detection may be useful in predicting the prognosis of NSCLC patients in the early stages of the disease, as well as in the development of a targeted therapy in these tumors.

Key Words : Telomerase reverse transcriptase; Carcinoma, Non-small cell lung; Prognosis

The incidence of non-small cell lung carcinoma (NSCLC), the largest subgroup of lung cancer, is increasing markedly in Korea, as well as in the rest of the world. The current management of NSCLC is guided principally by tumor stage. Patients with early-stage (I and II) tumors are treated via surgical resection with or without adjuvant chemotherapy, and stage III patients require combined modality approaches, which may include chemotherapy, radiation, and surgery.[1] Despite the significant progress that has been made in surgical and medical treatment, the overall prognosis is generally poor; the overall 5-year survival rate for NSCLC has remained at 15% or less, and NSCLC is the most prevalent cause of cancer-related deaths among Koreans.[2] In general, staging is the principal prognostic determinant in cases of NSCLC, but differing survival outcomes among patients within the same stage indicate that other tumor factors may also significantly affect prognosis. Recently, the use of molecular biological markers for diagnoses are under extensive investigation, in order to define a subset of patients with early-stage NSCLC as candidates for new investigational adjuvant therapies, a quest which eventually result in improvements in patient survival.[1,3]

Telomerase, a ribonucleoprotein enzyme, is suppressed in most normal human somatic cells, but is reactivated during tumorigenesis. As a continual loss of telomeric DNA is predicted to eventually limit cell proliferation, the activation of telomerase in cancer cells may be a pivotal step in the acquisition of cell immortalization, which occurs during tumor progression.[4] It has been recently determined that telomerase is comprised of a human telomerase, an RNA component (hTERC), a telomerase-associated protein (TEP1), and a reverse transcriptase subunit (hTERT).[5] Telomerase activity is closely correlated with the expression of hTERT mRNA, and it is believed that hTERT plays a key role in the mechanism underlying the regulation of telomerase activity in cellular immortalization and carcinogenesis.[6] Several reports have emphasized the use of non-commercial hTERT anti-

bodies for the determination of telomerase activity. Moreover, some studies have attempted to correlate hTERT protein expression and the biological status of tumors, via immunohistochemical methods.[7-11]

Some genetic alterations in the neoplasm have been determined to block cellular senescence, and to cause the transformation of a cell into an immortalized state. The findings of recent studies appear to suggest that the immortalizing process may be caused by a combination of hTERT and other regulatory factors, as the loss of tumor suppressor proteins, such as p16 and p53, is a prerequisite for cellular immortalization in some cell types.[12] Moreover, the induction of p16 and p53 in tumor cells will suppress the expression of hTERT in human cancer cells via transcriptional regulation.[13-15]

In this study we assessed the expression of the hTERT protein in patients with NSCLC via immunohistochemistry, and evaluated the association between the expression of this protein and the clinicopathological characteristics and expression of p16 and p53. Furthermore, we assessed the prognostic impact of hTERT protein in cases of NSCLC.

MATERIALS AND METHODS

Patients and samples

A total of 167 cases of primary NSCLCs of the lung were acquired from the Department of Hospital Pathology, at the College of Medicine, of the Catholic University of Korea, between 1 September 1994 and 30 August 2003. All of these patients had undergone a complete tumor resection. None of the patients had received any therapy prior to surgery. Histological classification and differentiation were assessed via light microscopy in accordance with WHO criteria. Clinical information was acquired via a computerized retrospective database of the tumor registry. After surgery, the clinical follow-up data for all patients were obtained. Survival time was measured as the time from the date of the initial surgery to the date of death. Those patients who died as the result of the surgery or from other causes were excluded.

Tissue microarray

In order to construct the tissue microarray block, small core biopsied samples were obtained from viable, morphologically representative areas of paraffin-embedded tumor tissues, then assembled on a recipient paraffin block. This was accomplished using a precision instrument (Micro Digital Co., Seoul, Korea). The biopsied core was 3.0 mm in diameter, which was sufficient for the evaluation of the morphological features of the analyzed tissues.[16] A total of 30 cores were assembled on a recipient paraffin block. After construction, 5 μm sections were cut, and hematoxyline-eosin staining was conducted on the initial slide in order to verify the histology.

Immunohistochemistry

Immunohistochemical staining was conducted on 5 μm sections of the tissue microarray blocks. The paraffin sections were mounted on superfrost glass slides, deparaffinized, and rehydrated in a graded series of ethanol, followed by microwave antigen retrieval. Endogeneous peroxidase activity was blocked using 0.3% hydrogen peroxide. The sections were incubated for 1 h or overnight at 4℃ using the following primary antibodies at 1:200 dilutions: anti-hTERT antibody (Novocastra Lab. Ltd, Newcastle, UK), anti-p16 antibody (DAKO Corporation, Carpinteria, CA, USA), and anti-p53 antibody (Zymed Laboratories Inc., San Francisco, CA, USA). Immunostaining was conducted using the rabbit or mouse DAKO ChemMate™, EnVision™, system, Peroxidase/DAB kit (DAKO). The sections were then counterstained with Meyer hematoxylin and were then dehydrated, cleared, and mounted. The positive controls were obtained from the tonsils for all studies.

Interpretation of immunohistochemical staining

Two independent pathologists who were blinded to the specific diagnosis and prognosis for each individual case interpreted the results of immunohistochemical staining. Cases evidencing distinct nuclear staining above any cytoplasmic background in >10% of the tumor cells were scored as positive for hTERT, p16, and p53.

Statistical analysis

Associations between categorical variables were analyzed using the SPSS software package, version 11.5 (Seoul, Korea). Two-sided p values were determined via χ^2 tests. Patient survival was analyzed using the Kaplan-Meier method with a log-rank test for univariate analysis. Multivariate analysis was conducted using the Cox regression model. The level of significance was set to 0.05.

RESULTS

Clinicopathological data

Among the 167 patients studied, 132 (79.0%) were men and 35 (21.0%) were women, with a mean age of 66 years (range, 19 to 89). In accordance with WHO criteria, the specimens included 86 (51.5%) squamous cell carcinomas and 81 (48.5%) adenocarcinomas. 26 (15.6%) of those tumors were classified as well differentiated, 108 (64.7%) as moderately differentiated, and 33 (19.8%) as poorly differentiated. All patients were staged at the time of their surgery, in accordance with the guidelines of the American Joint Committee on Cancer. 93 patients (55.7%) had stage I disease, 34 (20.4%) had stage II disease, and 40 (23.9 %) had stage III disease. Follow-up data were available for all patients, ranging from 0.6 to 115.7 months after the primary

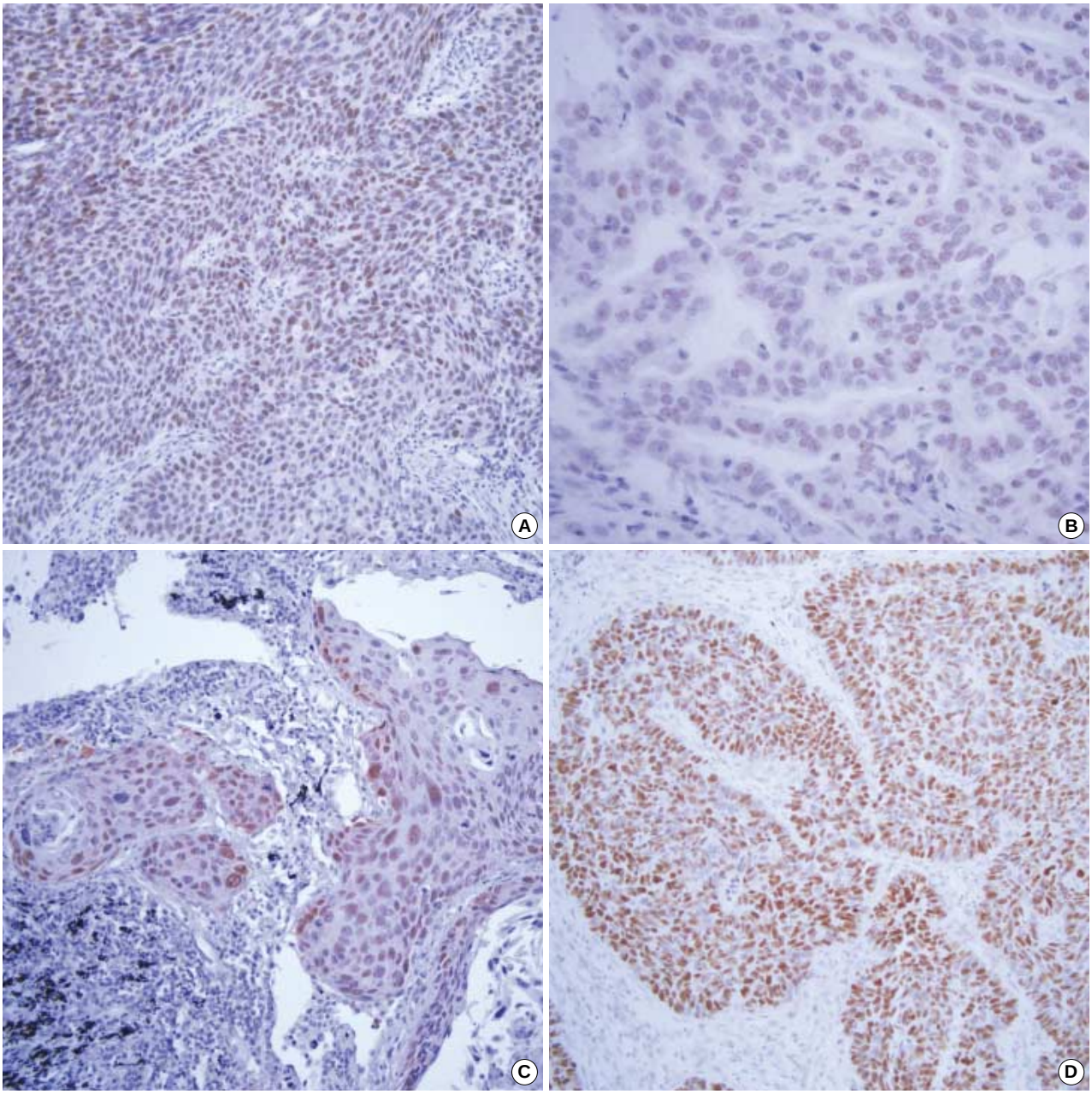

Fig. 1. The expression of hTERT (A, B), p16 (C) and p53 (D) in NSCLC. (A) hTERT is expressed in the nuclei of poorly differentiated squamous cell carcinoma. (B) hTERT is expressed in the nuclei of moderately differentiated adenocarcinoma. (C) p16 is expressed in the nuclei of moderately differentiated squamous cell carcinoma. (D) p53 is expressed in the nuclei of poorly differentiated adenocarcinoma.

operation (median follow-up time, 33.8 months). 68 patients died during the follow-up period, and 99 remained alive at the time of the study.

Immunohistochemical analysis

Of the 167 cases, hTERT expression was detected in 91 cases (54.5%) (Fig. 1A, B). Using higher magnification, the nuclear localization of hTERT expression was observed in most tumor cells evidencing positive expression, as has also been observed in the tonsils. As for gender, hTERT expression levels were found to be higher in males than in females (p=0.000). With regard to histological type, cases with squamous cell carcinomas (68.6%) evidenced significantly more frequent hTERT expression than cases with adenocarcinomas (39.5%) (p=0.000) (Table 1). Statistically significant associations of hTERT expression to the following clinicopathologic variables were not identified in the univariate analysis: age, differentiation, T factor, N factor, and stage.

p16 expression was positive in 44 cases (26.3%) among the 167 cases, and was present in the nuclei, as well as in the cytoplasm of the tumor cells (Fig. 1C). No statistically significant correlations could be observed between hTERT and p16 expression. A total of 74 of 167 cases (44.3%) displayed p53 immunoreactivity (Fig. 1D). The correlation between hTERT and p53 expression was found to be statistically significant (p=0.002), particularly in the squamous cell carcinoma cases (p=0.011) (Table 1).

Univariate and multivariate survival analyses for patients in NSCLC

Univariate analyses using the Kaplan-Meier method in NSCLC revealed that stage and lymph node metastasis were associated with patient survival (p=0.0166, p=0.0011, respectively) (Table 2, Fig. 2A). Neither the age nor gender of the patient, nor differentiation, was of prognostic significance. The T factor was of

Table 1. Relationship between hTERT expression and clinicopathological parameter and p16, pRb, p53 expressions in NSCLC

Variables	hTERT expression								
	NSCLC (n=167)			SCC (n=86)			AC (n=81)		
	n	Positive (%)	p value	n	Positive (%)	p value	n	Positive (%)	p value
Histologic type									
SCC	86	59 (68.6)	0.000						
AC	81	32 (39.5)							
Age									
<65	67	34 (50.7)	NS	29	19 (65.5)	NS	38	15 (39.5)	NS
≥65	100	57 (57.0)		57	40 (70.2)		43	17 (39.5)	
Gender									
Male	132	82 (62.1)	0.000	80	57 (71.3)	NS	52	25 (48.1)	0.035
Female	35	9 (25.7)		6	2 (33.3)		29	7 (24.1)	
Differentiation									
W	26	13 (50.0)		4	4 (100.0)		22	9 (40.9)	
M	108	60 (55.6)	NS	65	45 (69.2)	NS	43	15 (34.9)	NS
P	33	18 (54.5)		17	10 (58.8)		16	8 (50.0)	
pT									
T1-T2	136	72 (52.9)	NS	64	44 (68.8)	NS	72	28 (38.9)	NS
T3-T4	31	19 (61.3)		22	15 (68.2)		9	4 (44.4)	
pN									
N0	105	59 (56.2)	NS	56	39 (69.6)	NS	49	20 (40.8)	NS
N1-3	62	32 (51.6)		30	20 (66.7)		32	12 (37.5)	
Stage									
I-II	127	69 (54.3)	NS	64	46 (71.9)	NS	63	23 (36.5)	NS
III	40	22 (55.0)		22	13 (59.1)		18	9 (50.0)	
p16 expression									
Negative	123	69 (56.1)	NS	66	47 (71.2)	NS	57	22 (38.6)	NS
Positive	44	22 (50.0)		20	12 (60.0)		24	10 (41.7)	
p53 expression									
Negative	93	41 (44.1)	0.002	40	22 (55.0)	0.011	53	19 (35.8)	NS
Positive	74	50 (67.6)		46	37 (80.4)		28	13 (46.4)	

NSCLC, non-small cell lung carcinoma; SCC, squamous cell carcinoma; AC, adenocarcinoma, NS, not significant.

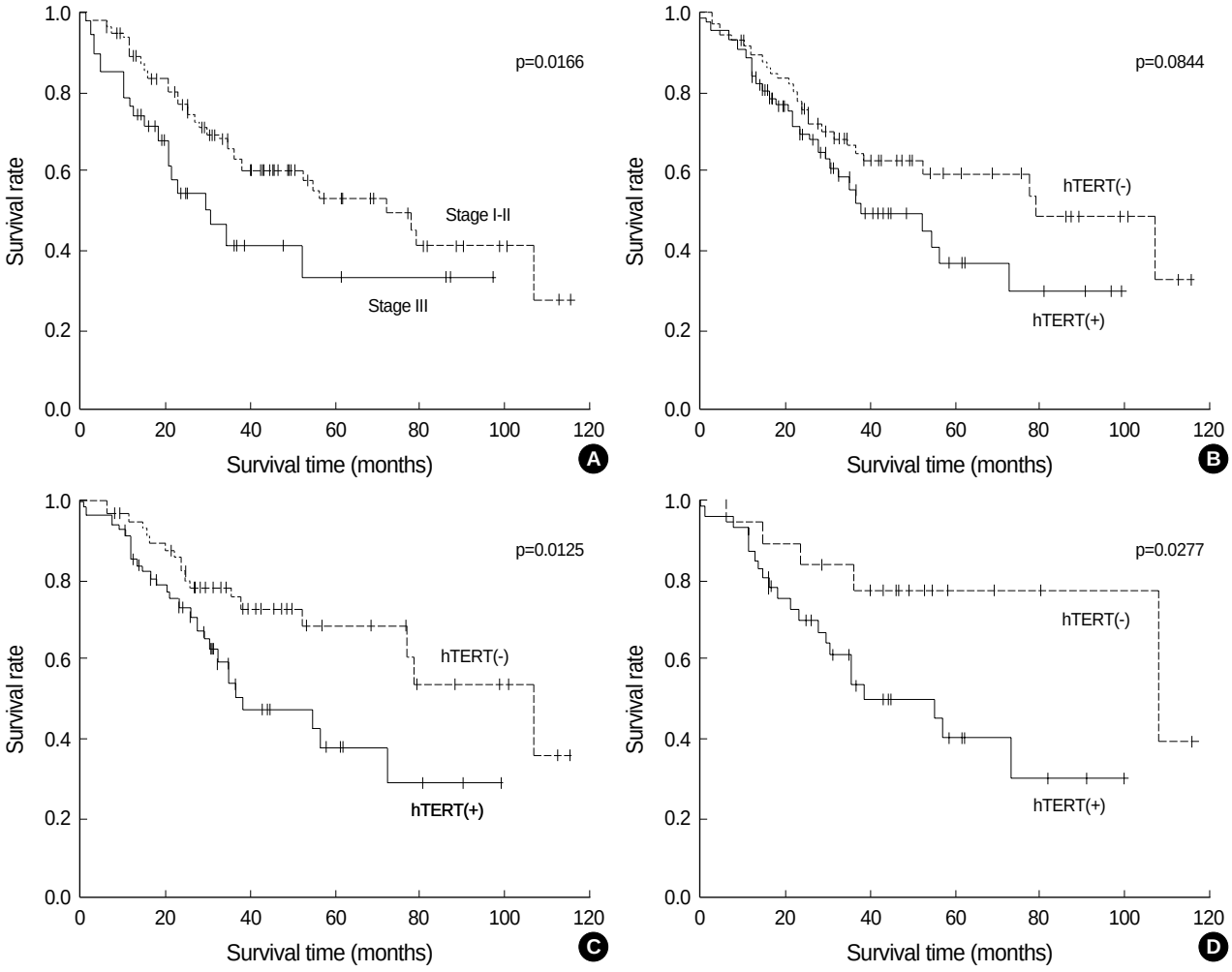

Fig. 2. Kaplan-Meier survival curves indicate prolonged survival in NSCLC patients with early stage (I and II) rather than advanced stage (III) (A). We analyzed the importance of hTERT expression in NSCLC patients with all stage and early stage. hTERT expression was a strong predictor for survival in stage I and II (C), especially in squamous cell carcinoma (D), but not in all stage (B).

borderline significance (p=0.052). In the squamous cell carcinoma cases, stage and N factor were also shown to be negative prognostic factors. However, in the adenocarcinoma cases, neither T factor, N factor, nor stage was of prognostic relevance. The correlation of hTERT expression with the overall survival of the NSCLC patients was also analyzed via the Kaplan-Meier method. However, hTERT expression was not determined to be associated with patient survival (p=0.0844) (Fig. 2B) not only in all of the NSCLC cases, but also in each histological subtype (Table 2).

We further analyzed the effects of hTERT expression on overall survival rates according to stage. In stage I and II NSCLC, patients evidencing hTERT expression in tumors evidenced significantly poorer survival as compared with patients who did not evidence hTERT expression (mean survival 52.0±5.6 vs 80.2± 6.5 months, p=0.0125) (Table 3, Fig. 2C). No significant associations were detected between hTERT expression and patient survival in patients with stage III disease (data not shown). With regard to histologic subtype, only in squamous cell carcinoma cases was hTERT expression shown to be a negative prognostic factor (p=0.0277) (Table 3, Fig. 2D).

We also evaluated the T factor, N factor, and hTERT expression against overall survival via multivariate analysis in NSCLC patients with early-stage disease (Table 4). In all cases of NSCLC, hTERT expression (p=0.006) in addition to the N factor (p= 0.020) was the strongest independent prognostic factor. In cases of squamous cell carcinoma, hTERT expression alone was the strongest independent prognostic factor (p=0.019) In the adenocarcinoma cases, the T factor and N factor were independent prognostic factors (p=0.011, p=0.014, respectively).

Table 2. Univariate survival analysis (Kaplan-Meier) in all stage NSCLC patients

Variables	NSCLC (n=167)		SCC (n=86)		AC (n=81)	
	n	p value	n	p value	n	p value
Age (years)						
<65	67	NS	29	NS	38	NS
≥65	100		57		43	
Gender						
Male	132	NS	80	NS	52	NS
Female	35		6		29	
Differentiation						
W	26		4		22	
M	108	NS	65	NS	43	NS
P	33		17		16	
pT						
T1-T2	136	0.0520	64	NS	72	NS
T3-T4	31		22		9	
pN						
N0	105	0.0011	56	0.0128	49	NS
N1-3	62		30		32	
Stage						
I-II	127	0.0166	64	0.0280	63	NS
III	40		22		18	
HTERT expression						
Negative	76	0.0844	27	0.0872	49	0.7371
Positive	91		59		32	

Table 3. Univariate survival analysis (Kaplan-Meier) in early-stage NSCLC patients

Variables	NSCLC (n=127)		SCC (n=64)		AC (n=63)	
	n	p value	n	p value	n	p value
Age (years)						
<65	42	NS	17	NS	25	NS
≥65	85		47		38	
Gender						
Male	104	NS	60	NS	44	NS
Female	23		4		19	
Differentiation						
W	20		3		17	
M	82	NS	49	NS	33	NS
P	25		12		13	
pT						
T1-T2	116	NS	58	NS	60	0.0205
T3	9		6		3	
pN						
N0	102	0.0288	54	NS	48	0.0651
N1-3	25		10		15	
HTERT expression						
Negative	58	0.0125	18	0.0277	40	NS
Positive	69		64		23	

Table 4. Cox regression multivariate analysis of overall survival time in early-stage NSCLC patients

Variables	NSCLC (n=127)		SCC (n=64)		AC (n=63)	
	RR (95% CI)	p value	RR (95% CI)	p value	RR (95% CI)	p value
pT (T1-2/T3)	1.059 (0.613-1.827)	NS	0.753 (0.360-1.578)	NS	4.015 (1.680-9.596)	0.011
pN (N0/N1-3)	2.185 (1.171-4.077)	0.020	1.878 (0.742-4.751)	NS	3.433 (1.325-8.893)	0.014
hTERT expression (negative/positive)	2.285 (1.246-4.190)	0.006	3.133 (1.074-9.145)	0.019	2.506 (0.929-6.757)	0.075

RR, risk ratio; CI, confidence interval.

DISCUSSION

Three major components of human telomerase--, the RNA component (hTERC), the telomerase-associated protein (TEP1), and the reverse transcriptase subunit (hTERT)-- have been recently cloned.[5] Among these, hTERT is located on chromosome 5p 15.33 and harbors several sequence motifs characteristic of the catalytic region of reverse transcriptase.[5] In recent reconstitution experiments, the expression of hTERT, but not of hTERC and TEP1 parallels telomerase activity as detected by the telomeric repeat amplification protocol (TRAP) assay. The *in vitro* transformation of telomerase-negative normal cells using defined genetic elements usually requires hTERT expression.[17] These findings strongly indicate that the activity of hTERT is the rate-limiting step in telomerase activity and cellular immortalization. For NSCLC, several reports have indicated a high concordance between hTERT expression and telomerase activity.[18,19] However, in patients with NSCLC, a conclusion regarding the prognostic value of hTERT expression remains unclear. Some studies have determined that telomerase activity was a poor prognostic factor in NSCLC patients, but we detected no relationship between hTERT expression and clinicopathological features, including patient prognosis.[18,19] However, other studies have shown that hTERT expression was an independent prognostic factor for NSCLC patients.[20,21] In comparison with previous studies, we have analyzed the association between hTERT expression and patient survival in accordance with stage and histologic subtype.

We have determined that hTERT protein levels are increased in 54.5% (91/167) of NSCLC patients, and that protein expression was principally expressed in the nuclei in cancer cells, in

accordance with previous reports.[7,9] HTERT mRNA expression, as detected by RT-PCR and *in situ* hybridization, was detected primarily in the cytoplasm of cancer cells. These results show that the hTERT protein, which is generated by the transcription of hTERT mRNA in the cytoplasm, becomes localized within the nucleus, and fuctions as an intranuclear protein.[7] When we analyzed the association between hTERT expression and patient prognosis, we detected a trend that patients with hTERT-positive tumors evidenced shorter survival times than those with hTERT-negative tumors in all stages of NSCLC, although this correlation was not statistically significant. The disease stage at presentation has been established to be the major determinant of survival in NSCLC. Stage III disease is characterized by the presence of metastatic lymph nodes within the mediastinum. These patients already have a far less favorable prognosis than those with stage I and II disease, and the likelihood of occult metastasis is high. Therefore, we limited our survival analysis to patients with disease stages I and II, the prognosis of which is quite difficult. In early stage NSCLC, we have determined that hTERT expression is an independent predictor of shortened patient survival, as is shown by both univariate and multivariate analysis. Particularly with regard to histologic subtype, hTERT expression was the strongest independent prognostic factor in the cases of squamous cell carcinoma. Our results show that hTERT appears to be an important prognostic factor, which should be validated in future prospective multi-institutional trials of adjuvant therapy for NSCLC patients with early stage disease, who suffer high- risk of cancer-related events, including recurrence or metastasis.

Recently, a two-stage model was introduced to explain the escape from senescence of cultured human cells.[22,23] Cells must first overcome a proliferative block (M1) allowing for further cell divisions before widespread death ensues during 'crisis' (M2). Cell clones that survive the crisis stage are often tumorigenic and become immortalized. At the molecular level, it has been proposed that M1 may be reflected in the inactivation of p16/pRb or p53 and at the M2 stage, telomere stabilization by telomerase reactivation might be required for further cell survival.[22,23]

We determined that, in the majority of cases, 123 cases (73.7 %) were p16 negative, which indicated the loss of the p16 protein. 74 of 167 cases (44.3%) evidenced p53 abnormality. In these tumors, the altered expression of their tumor suppressors might enable a tumor to bypass the M1 stage. The reactivation of telomerase in cases of tumors evidencing hTERT expression might be capable of passing through the M2 stage of immortalization. However, no correlation between hTERT and p16 expression was detected in this study. This would show that the loss of p16 expression is not a critical requirement for the activation of telomerase in NSCLC, despite recent evidence that p16 is capable of inhibiting telomerase activity.[24,25]

When searching for a correlation between hTERT and p53 expression, we observed that hTERT expression was correlated positively with the expression of p53. The wild- type p53 protein has a short half-life of approximately 20 min, and generally cannot be detected via immunohistochemical methods. However, a p53 gene mutation or p53 interacting with an oncoprotein may stabilize the p53 protein, and may result in altered p53 expression, which can be detected by immunohistochemical methods.[26] It has been shown that telomerase activation was accompanied by the loss or mutation of the remaining wild-type p53 allele in Li-Fraumeni syndrome skin fibroblasts, which is characterized by a heterozygous germline mutation within the p53 gene.[27] Other studies have shown that wild-type p53 represses telomerase activity via the downregulation of hTERT transcription.[14,15] These findings indicate the existence of a p53-dependent regulatory pathway for hTERT/telomerase control, and an inhibitory effect against telomerase may thus be a novel function of the antitumor activity of p53.

In conclusion, our results show that hTERT expression may contribute to the progression of NSCLC, and its detection may function as a new prognostic marker for NSCLC patients with early-stage disease. The immunohistochemical detection of hTERT expression may be used to distinguish patients with poor prognosis, and to potentially guide the regimen of adjuvant chemotherapy for NSCLC patients with early- stage disease.

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