hTERT Expression Is a Prognostic Factor of Survival in Patients with Stage I Non-Small Cell Lung Cancer

Luo Wang, Jean-Charles Soria, Bonnie L. Kemp, Diane D. Liu, Li Mao, and Fadlo R. Khuri

INTRODUCTION

Lung cancer retains the leading position in cancer-related deaths in the United States. In 2002, it is estimated that there will be 154,900 deaths and 169,400 new cases from lung and bronchial cancer in the United States, compared with 156,900 deaths and 164,100 new cases in 2000 (1). NSCLC comprises more than 80% of lung cancers, and complete surgical resection of primary tumors in early-stage disease is the only potentially curative treatment. For patients with stage I NSCLC (about 17% of all patients with NSCLC), the average 5-year survival rate is about 60%. Adjuvant cytotoxic chemotherapy has been proposed and evaluated in the setting of NSCLC, and it offers limited hope of improving prognosis (2). One area of intense research on early-stage NSCLC is the identification of molecular markers to complement TNM staging to fully assess the prognosis of patients and to evaluate the effects of novel chemotherapy agents and regimens (3). Such prognostic markers include a wide variety of protein molecular markers that can be classified by different antibodies as molecular genetic markers, metastatic propensity markers, differentiation markers, and proliferation markers (3). Other markers have been evaluated at the mRNA level including retinoic acid receptor-β, cyclooxygenase-2, vascular endothelial growth factor, MMP-2 and -9, E-cadherin, angiopoietin-2, and CD44. These markers have been associated with clinicopathological variables and survival time in patients with NSCLC (4–8).

Telomerase is a ribonucleoprotein enzyme that lengthens chromosome ends that have been shortened during successive cycles of cell division (9). Telomerase is expressed in up to 85% of NSCLC (10, 11), and its activation plays a critical role in tumorigenesis by sustaining cellular immortality (12, 13). Hahn et al. (14) proved that disruption of the intracellular pathways regulated by large T antigen, oncogenic ras, and telomerase suffices to create a human tumor cell. The components of human telomerase include an RNA subunit (hTERC), a catalytic protein subunit (hTERT), and other telomerase-associated proteins (15). It has been shown that the expression pattern of hTERT is closely associated with telomerase activity (16–18). The recent development of ISH techniques that can reliably detect hTERT mRNA has made it possible to examine the expression of this critical telomerase component at the single-cell level (18–21). In our previous study (22), we evaluated hTERT expression in bronchial biopsy samples and found that it was a frequent event and appeared at a very early stage in cigarette smoking-induced lung carcinogenesis, making it clearer that telomerase plays a critical role in tumorigenesis. Because we have established a reliable ISH technique for detecting hTERT mRNA expression in paraffin-embedded tissue, we decided to evaluate the prognostic value of hTERT in a relatively homogeneous tumor, in a population of 153 patients with stage I NSCLC.

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3 The abbreviations used are: NSCLC, non-small cell lung cancer; hTERT, human telomerase reverse transcriptase subunit; ISH, in situ hybridization; MMP, matrix metalloprotease; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; TRAP, telomeric repeat amplification protocol; TNM, tumor node metastasis; CI, confidence interval; SCC, squamous cell carcinoma.
MATERIALS AND METHODS

Clinical Samples and Preparation of Slides. Five hundred ninety-five consecutive patients with stage I NSCLC underwent definitive surgical resection, defined as a lobectomy or a pneumonectomy, from 1975 to 1993 at The University of Texas M. D. Anderson Cancer Center. We retrospectively examined 153 cases for which both tissue samples as well as data from a median follow-up period of more than 5 years were available. All available tissue blocks for each patient were reviewed for the presence of tumor by a thoracic pathologist (B. L. K.). To prevent RNA degradation in the tissue blocks during sectioning, we used glass slides that were pretreated with diethylpyrocarbonate-treated water (Sigma Chemical Co., St. Louis, MO) and coated with poly-lysine (Sigma Chemical Co.). The patient population was identified through a search of the Tumor Registry Database maintained by the Department of Medical Informatics at M. D. Anderson Cancer Center. The study was reviewed and approved by the institution’s Surveillance Committee to allow us to obtain the tissue blocks and all pertinent follow-up information.

Generation of Single-Strand-specific Riboprobes. The riboprobe used in the present study, a 430-bp EcoRV-BamHI fragment of the full-length hTERT cDNA, is identical to the one initially reported by Kolquist et al. in 1998 (18). We have successfully tested this probe in 532 paraffin-embedded sections of bronchial origin, in a previous study (22). Although larger than the classical 50–300-bp probes usually developed for ISH, this probe remains well in the range of previous probes used to evaluate hTERT by ISH (18–22). Part of exon 1 from hnRNP A1 was used as a control to verify sample quality.

Both cDNA fragments were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). The single-strand-specific riboprobes were generated by using in vitro transcription. In brief, the plasmid was linearized with EcoRV and then transcribed in vitro with SP6 RNA polymerase (Promega, Madison, WI) using a DIG RNA labeling kit (Roche Diagnostics, Inc., Indianapolis, IN). The resulting digoxigenin-labeled RNA probe was mixed with RNase inhibitor (Roche Diagnostics, Inc.) and stored in aliquots at −80°C.

RNA ISH. ISH was performed as described previously (22). Briefly, the sections were deparaffinized in xylene and then gradually rehydrated in decreasing concentrations of ethanol. They were then treated with 2.5 μg/ml proteinase K (Roche Diagnostics, Inc.), post-fixed in 4% paraformaldehyde, and acetylated in 0.25% acetic anhydride/0.1 M triethanolamine (Sigma Chemical Co.). After dehydrating in increasing concentrations of ethanol and air-drying, the sections were hybridized with the probe at 42°C for 4 h by incubating in hybridization buffer [400–800 ng/ml of either hTERT or hnRNP A1 riboprobe, 10% 20× SSC, 50% deionized formamide, 5% dextran sulfate, 2% 100× Denhardt’s solution (2% Ficoll 400, 2% povidone, and 2% BSA), 10 mM DTT, 250 μg/ml preadenaturated salmon sperm DNA, and 200 μg/ml yeast tRNA]. The sections were then washed two times for 5 min in 2× SSC and then for 2 h in 2× SSC containing 0.05% Triton X-100 and 2% normal sheep serum (Sigma Chemical Co.) with agitation at room temperature. After being briefly rinsed in buffer 1 [0.1 M maleic acid and 0.15 M NaCl (pH 7.5)], the sections were washed in buffer 1 containing 0.3% Triton X-100 and 2% normal sheep serum for another 30 min at room temperature.

Detection was performed using the DIG Nucleic Acid Detection Kit (Roche Diagnostics, Inc.) according to the manufacturer’s directions. Anti-DIG alkaline phosphatase-conjugated antibody was diluted 1:500. Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as chromogens. Slides were then rinsed in TE buffer [10 mM Tris-HCl/1 mM EDTA (pH 8)] and mounted with Aqua-Mount medium (Fisher, Houston, TX).

Determination of Positive hTERT Expression. As reported by Falchetti et al. (21), only slides displaying a clear cytoplasmic signal could be considered as positive. More specifically, our slides were rated as positive if such a definite and clear signal was present in at least two large areas (×200 magnification) on the slide. Slides with faint signal, the absence of signal, or only focal positivity were considered to be negative. We did not grade the intensity of the hybridization signals.

To confirm RNA preservation in hTERT-negative slides, we randomly selected 33 such negative slides and detected the expression of the major splicing factor hnRNP A1. Because hnRNP A1 is one of the most abundant splicing factors in human cells, this probe was a good control to check mRNA quality.

Statistical Analysis. All statistical analyses were performed using SAS software (version 6.12; SAS Institute, Inc., Cary, NC). Overall, disease-specific and disease-free survival rates were calculated using the Kaplan-Meier method. All survival times were calculated from the date of surgery. The overall survival statistic accounted for all deaths (cancer related or not). Disease-specific survival time was calculated from the date of surgery to death from cancer-related causes. Disease-free survival time was calculated from the date of surgery to relapse or death from cancer-related causes. The χ² test was used to test the association between two categorical variables. The Wilcoxon rank-sum test was used for differences in median of age. We used the Cox proportional hazards model for univariate analysis to evaluate the association between survival time and risk factors and for multivariate analysis to model the risks of hTERT expression on survival time, with adjustment for clinical and histopathological parameters (age, sex, race, tumor histology, tumor size). All Ps were determined by two-sided tests. P values less than 0.05 were considered statistically significant.

RESULTS

A total of 153 cases that had adequate tumor specimen and ≥5-year follow-up information were analyzed for hTERT mRNA expression in this study. The study population consisted of 115 men and 38 women; 136 patients were white, and 17 patients were of other ethnicities (Table 1). Patient ages ranged from 37–85 years old, with a median age of 63.4 ± 9.2 years old. Histological subtypes included 66 cases of SCC, 59 cases of adenocarcinoma, 12 cases of bronchioalveolar carcinoma, 5 cases of large cell carcinoma, 5 cases of adenosquamous carcinoma, and 6 unclassified cases. Thirty-nine patients died of lung cancer. The other patients died of heart disease (21 cases), respiratory diseases (15 cases), other organ failures (6 cases), and unknown causes (24 cases). The probability of 5-year over-
Between age, sex, tumor size (T1N0M0 patients is shown in Table 1. There was no statistical significance and the general clinicopathological characteristics of the pa-

The association between hTERT expression show any difference between the positive rates of slides by year in the different years of surgery, and statistical analysis did not

33.3% (51 cases). The positive slides were randomly distributed

The hybridization signal for hTERT mRNA in the tumorous area of positive

were negative for hTERT mRNA (i.e.

had no hybridization

were hTERT negative (Table 1). Fig. 3A shows the overall survival curves analyzed using the Kaplan-Meier method. Patients with tumors that were hTERT positive had a shorter survival time than did patients with tumors that were hTERT negative (P = 0.04; log-rank test). The 5-year overall survival rate for patients whose tumors were hTERT positive was 42.7% (95% CI, 30.9–58.8%) and 62.9% (95% CI, 54.1–73.1%) for patients whose tumors were hTERT negative (Table 1). Fig. 3B shows that patients with positive hTERT expression had significantly shorter disease-specific survival times than did patients with negative hTERT expression (P = 0.03; log-rank test). A comparison of disease-free survival curves in hTERT-negative and hTERT-positive patients yielded similar results (P = 0.02; log-rank test; Fig. 3C).

The univariate Cox proportional hazards model was used to evaluate the association between hTERT expression, clinicopathological variables (age, sex, race, histological subtype, TNM stage), and survival time. Table 2 shows the results on disease-specific survival time. In a multivariate Cox proportional hazards model, among all clinicopathological variables, hTERT expression was the only significant independent prognostic indicator for disease-specific survival.

### DISCUSSION

Numerous prognostic factors have been identified in patients with early-stage NSCLC that might enable classification of such patients into different subsets corresponding to different risks of recurrence following complete resection. Most of the markers are proteins that can be detected by immunohistochemistry assays based on the antigen-antibody reaction. These mark-

We subsequently analyzed the relationship between hTERT expression and length of survival. The median follow-up time for the patient population was 10.5 years. Fig. 3A shows the overall survival curves analyzed using the Kaplan-Meier method. Patients with tumors that were hTERT positive had a shorter survival time than did patients with tumors that were hTERT negative (P = 0.04; log-rank test). The 5-year overall survival rate for patients whose tumors were hTERT positive was 42.7% (95% CI, 30.9–58.8%) and 62.9% (95% CI, 54.1–73.1%) for patients whose tumors were hTERT negative (Table 1). Fig. 3B shows that patients with positive hTERT expression had significantly shorter disease-specific survival times than did patients with negative hTERT expression (P = 0.03; log-rank test). A comparison of disease-free survival curves in hTERT-negative and hTERT-positive patients yielded similar results (P = 0.02; log-rank test; Fig. 3C).

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**Table 1** hTERT expression status in stage I NSCLC tumors according to clinicopathological features of patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total no. of patients (n = 153)</th>
<th>Positive (n = 51)</th>
<th>Negative (n = 102)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>63.4 ± 9.2</td>
<td>64.3 ± 9.5</td>
<td>62.9 ± 9.1</td>
<td>0.38</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>115</td>
<td>40</td>
<td>75</td>
<td>0.51</td>
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<tr>
<td>Female</td>
<td>38</td>
<td>11</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>White</td>
<td>136</td>
<td>42</td>
<td>94</td>
<td>0.09</td>
</tr>
<tr>
<td>Other</td>
<td>17</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>137</td>
<td>47</td>
<td>90</td>
<td>1b</td>
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<tr>
<td>No</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td></td>
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<tr>
<td>Unknown</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Tumor histology</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>SCC</td>
<td>66</td>
<td>27</td>
<td>39</td>
<td>0.08</td>
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<tr>
<td>Adenocarcinoma and others</td>
<td>87</td>
<td>24</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1N0M0</td>
<td>75</td>
<td>27</td>
<td>48</td>
<td>0.49</td>
</tr>
<tr>
<td>T2N0M0</td>
<td>78</td>
<td>24</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>5-year overall survival rate (95% CI)</td>
<td>56.1% (48.7%, 64.6%)</td>
<td>42.7% (30.9%, 58.8%)</td>
<td>62.9% (54.1%, 73.1%)</td>
<td></td>
</tr>
</tbody>
</table>

*This number included 36 cases in which the signal was faint or focal.

b The P was calculated to compare smoking and nonsmoking patients.

By using the hTERT riboprobe, we detected diffuse and clear hTERT mRNA expression in tumor cell nests as well as in some infiltrating tumor lymphocytes (Fig. 1). In pilot experiments, the positive hybridization signal was always cytoplasmic and was abrogated by RNase treatment of the sections before hybridization with the riboprobe, suggesting that the signal was related to the presence of hTERT mRNA. The hybridization signal for hTERT mRNA in the tumorous area of positive samples ranged in intensity from low or moderate to strongly positive and was detectable in the vast majority of cells examined (Fig. 1). Interestingly, we found hTERT-positive cases in all of the histological subtypes we tested (Fig. 1). Slides that were negative for hTERT mRNA (i.e., that had no hybridization signal or only focal positivity) also contained different histological subtypes (Fig. 2). We clearly detected hnRNP A1 mRNA, used as a positive control, in all 33 of the randomly selected hTERT-negative slides, therefore ruling out false-negativity related to RNA degradation (Fig. 2).

Among these 153 cases screened for hTERT expression by ISH, the percentage of tumors that were hTERT positive was 33.3% (51 cases). The positive slides were randomly distributed in the different years of surgery, and statistical analysis did not show any difference between the positive rates of slides by year (data not shown). The association between hTERT expression and the general clinicopathological characteristics of the patients is shown in Table 1. There was no statistical significance between age, sex, tumor size (T1N0M0 versus T2N0M0), and smoking status between the hTERT-positive cases and the hTERT-negative cases. Only seven patients were nonsmokers, thereby preventing any definitive conclusion regarding the association between hTERT expression and smoking. There was a trend, but no statistical significance, toward more cases with positive expression of hTERT in Caucasians (P = 0.09).
2/p185, bcl-2 protein, and Rb protein; metastatic propensity markers such as CK18 protein, cathepsin B protein, factor VIII and type IV collagen; differentiation markers such as the ABH blood group antigen and the Lewis-related antigen; and proliferation markers such as Ki-67 nuclear antigen and proliferating cell nuclear antigen (3).

Because the major value of prognostic markers is to guide postresection treatment in early-stage NSCLC, the ability to identify patients with a high risk of cancer-related events such as recurrence or metastasis will help to determine whether adjuvant therapy is needed and to evaluate its effect. However, no conclusions have been reached about which marker or markers are better for forecasting patients' outcomes. Therefore, one of the major current interests in this field is to evaluate other novel

Fig. 1  hTERT expression detected by ISH in an adenocarcinoma (A), a SCC (B), and a large cell carcinoma (C). Magnification, ×100. Panels D, E, and F represent Hematoxylin and Eosin stained samples from the same tumors and adjacent slices to panels A, B, and C.

Fig. 2  Sample quality detected by ISH with hnRNP A1 as the control in hTERT-negative cases. Magnification, ×200.
markers. Some effort has been put forth to test mRNA markers such as retinoic acid receptor-β, cyclooxygenase-2, vascular endothelial growth factor, MMP-2, MMP-9, E-cadherin, angiopoietin-2, and CD44 (4–8).

Telomerase activity has been described as an independent marker of poor prognostic in different human tumors such as neuroblastoma, gastric cancer, breast cancer, colon cancer, cervical cancer, and meningioma (23–28). In patients with NSCLC, a conclusion regarding the prognostic value of telomerase activity remains unclear. Hiyama et al. (29) first observed a high level of telomerase activity in primary tumors and corresponding metastatic lesions. However, Albanell et al. (30) found only a weak association between telomerase activity and unfavorable prognosis in a mixed population of 99 patients with stages I–IV NSCLC. Komiya et al. (31) examined the expression of hTERT mRNA in tumor specimens from 68 patients by using RT-PCR and did not find a correlation between hTERT status and any common clinical features, except age. However, Marchetti et al. (32) evaluated the activity of the telomerase enzyme detected by the TRAP assay in tumors and adjacent noncancerous lung tissue samples obtained from 107 consecutive patients with pathological stage I operable NSCLC. They found telomerase activity in 66 of 107 tumors but in none of the corresponding adjacent noncancerous lung tissue samples. They also found a statistically significant association between telomerase activity and both disease-free and overall survival times. Kumaki et al. (33), Arinaga et al. (34), and Satoshi et al. (35) reported different conclusions.

We considered that such contradictions in the literature could be attributed to analysis of heterogeneous populations with few patients with stage I NSCLC, and to the different techniques used to detect telomerase activity or expression. To address these issues, we decided to use ISH to measure the mRNA expression level of hTERT in a large population of 153 patients with stage I NSCLC for which complete follow-up information was available. Because the tumor samples had been stored for a long time (8–26 years), we anticipated that sample quality would be the first major problem in getting a correct signal. The second major issue for successful ISH is the probe. A single-strand riboprobe can yield a more specific signal than that of a standard double-strand cDNA probe labeled mostly by the random-primer method. The size and sequence of the riboprobe are also very important for successful ISH. The major component of human telomerase is hTERT, and it has been proven that the expression pattern of hTERT is closely associated with actual telomerase activity (16–18). On the basis of the analysis of the hTERT genomic sequence, we selected the most conserved region, from exon 7 to exon 12, as the probe, which also corresponded to the catalytic domain of this enzyme. The result is shown in Fig. 1. We are convinced that the hTERT ISH is a reliable technique. It can possibly be developed as a clinical assay, and, in fact, in a clinical trial of n-(4-hydroxyphenyl) retinamide, a compound derived from 13-cis-retinoic acid, we successfully used ISH to evaluate its effect on hTERT expression in the bronchial epithelium of smokers (22).

One major concern generated by our results is the low percentage (33%) of hTERT-positive cases reported in contrast to previous studies reporting telomerase activity in up to 85% of NSCLCs. We believe that this apparent discrepancy can be explained by the specific technique (ISH) and the strict criteria used in our study in comparison to the very sensitive TRAP assay. It is very important to point out that the TRAP assay does not account for tumor heterogeneity, and, therefore, tumors with few telomerase-positive cells appear telomerase positive in the same way as tumors in which most cells are telomerase positive. Furthermore, most previous studies on telomerase have been carried out in heterogeneous populations including patients with

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### Table 2

A univariate Cox proportional hazards model applied to disease-specific survival time

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>1.0</td>
<td>0.98–1.05</td>
<td>0.51</td>
</tr>
<tr>
<td>Sex (male or female)</td>
<td>1.1</td>
<td>0.57–2.14</td>
<td>0.77</td>
</tr>
<tr>
<td>Race (white or other)</td>
<td>0.56</td>
<td>0.25–1.25</td>
<td>0.15</td>
</tr>
<tr>
<td>Histological subtype (SCC or other)</td>
<td>0.76</td>
<td>0.41–1.42</td>
<td>0.39</td>
</tr>
<tr>
<td>Tumor size (T1 or T2)</td>
<td>1.03</td>
<td>0.57–1.86</td>
<td>0.92</td>
</tr>
<tr>
<td>hTERT (+ or −)</td>
<td>1.90</td>
<td>1.04–3.45</td>
<td>0.036</td>
</tr>
</tbody>
</table>

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Fig. 3 Survival analysis of 153 patients with stage I NSCLC based on the classification of hTERT-positive and -negative mRNA expression. A, overall length of survival; B, disease-specific survival time; C, length of disease-free survival.
hTERT Expression as a Prognostic Marker for NSCLC

stages I–IV disease (29–31). Marchetti et al. (32) reported a hTERT positivity rate of 66% by RT-PCR in stage I NSCLC. Falchetti et al. (21) investigated the expression of hTERT in 34 samples from patients with primary de novo glioblastoma multiforme by ISH, RT-PCR, TRAP activity assay, and telomere restriction fragment Southern blotting. They found that 60% of the cases were hTERT positive by ISH and could be classified into two groups: those with diffuse and those with focal hTERT expression. However, some studies have shown that telomerase activity can be detected at high, moderate, and low levels (26, 28). In cell lines, we found that the ISH signal generally corresponded to the level of telomerase activity detected by TRAP assay (data not shown). We considered that the focal hTERT expression does not seem to represent strong telomerase activity and decided to classify the cases with hTERT focal expression as negative.

In this study, even with our criteria, which are stricter than those used in other studies, a definite association exists between hTERT expression and all of the clinically relevant outcomes. This strongly supports our notion that substantial hTERT mRNA expression could be an ideal marker for assessing prognosis of patients with early-stage NSCLC and evaluating the effect of new chemotherapeutic agents.

In conclusion, based on a large population of patients with stage I NSCLC, our results indicate that hTERT mRNA expression is associated with malignant tumor progression, thereby making it a potentially suitable prognostic marker. ISH of hTERT expression may be used to distinguish patients with poor prognosis and to potentially guide the regimen of adjuvant chemotherapy for patients with early-stage NSCLCs. Nevertheless, confirmatory studies by independent groups, using ISH or alternative techniques (RT-PCR, TRAP), are necessary.

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