Prognostic Impact of Telomerase Activity in Non-Small Cell Lung Cancers

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Objective
To evaluate the clinical significance of telomerase activity, particularly in terms of prognostic impact, in non-small cell lung cancer (NSCLC).

Summary Background Data
Telomerase activity has been found in various tissues. The activation of telomerase is considered necessary for the immortalization of human tumor cells, including NSCLC.

Methods
The authors studied 103 NSCLC specimens using a polymerase chain reaction based on a telomeric repeat amplification protocol assay.

Results
Telomerase activity was detected in 85 (82.5%) of 103 NSCLC specimens but in none of the paired normal lung tissue specimens. More cases of positive telomerase activity were observed in the group with advanced disease and in the group with poorly differentiated tumors. Such factors as the mean age at surgery, sex, smoking, histologic type, and size of tumor extension did not correlate with the telomerase activity. The Kaplan-Meier survival curves in all patients with NSCLC demonstrated that patients with telomerase-positive tumors survived for a significantly shorter period than those with a telomerase-negative tumor ($p = 0.0058$). According to a multivariate analysis, telomerase activity was identified as an independent prognostic factor ($RR = 8.62, p = 0.035$).

Conclusions
Telomerase activity was one of the most important prognostic factors in patients with NSCLC, and its potential prognostic implication was independent of tumor stage.

Telomeres are the specialized structures at the end of eukaryotic chromosomes. In humans and all other vertebrates, telomeric DNA consists of tandem repeats of the highly conserved G-rich sequence TTAGGG.1–3 These repeats are thought to protect genomic DNA from degradation and deleterious recombination events.1,2 Normal human somatic cells lose 50 to 200 bp of terminal telomeric DNA with each round of replication1–6 because of the inability of DNA polymerase to replicate fully the ends of a linear DNA template6–8 and presumably because of the lack of telomerase activity.9,10 Telomerase is a ribonucleoprotein enzyme that uses its own integral RNA as a template for synthesis on the TTAGGG telomeric repeats to compensate for the normal loss of telomeric repeats during cell divisions.11 Recently, a highly sensitive polymerase chain reaction (PCR)-based telomerase assay called the telomeric amplification protocol (TRAP) was developed to detect telomerase activity.10,12 Using this method, telomerase activity has been found in various tissues. In cultured cells, 98% of immortal and no mortal cell populations were found to express telomerase activity.10 In vivo, telomerase activity appears to be repressed in somatic cells and tissue, except for the fact that some reproductive somatic cells revealed a low level of telomerase activity, such as epidermal cells,13 intestinal mucosal cells,14 peripheral blood monocytes, and hematopoietic progenitor cells.15 In tumor tissue, telomerase activity was detected in 94% of neuroblastoma,16 93% of colorectal cancer,17 85% of gastric cancer,18 and 85% of hepatocellular carcinoma specimens.19 In lung cancer, telomerase activity was detected in almost all small cell lung cancer specimens and in approximately 80% of non-small cell lung cancer (NSCLC) specimens.20 There has been no previous report regarding the telomerase activity.
activity and patient prognosis in NSCLC. In this study, we examined the clinical significance of the telomerase activity, especially regarding its prognostic impact in NSCLC.

MATERIALS AND METHODS

Tissue Samples and Patients

From July 1991 until April 1996, 291 consecutive patients with NSCLC underwent radical surgery of the primary tumor with the dissection of hilar and mediastinal lymph nodes (systematic nodal dissection) at the Department of Surgery II, University of Occupational and Environmental Health, Japan. Of these 291 patients, we obtained adequate volumes of frozen tumor tissues and paired normal lung tissues suitable for analysis from 103 patients. The histologic sections were reviewed by two or three pathologists as a routine procedure at our institute without knowledge of each patient’s molecular biologic or clinical data. The degree of tumor differentiation was also interpreted at the same time. The median follow-up period was 924 days. The patients ranged in age from 26 to 83 years (mean 64 years) and included 78 men and 25 women. The histologic types included 54 adenocarcinomas, 43 squamous cell carcinomas, 4 large cell carcinomas, and 2 others.

The pathologic stage was classified according to the International System for Staging Lung Cancer: stage IA in 16 patients, stage IB in 25 patients, stage IIA in 1 patient, stage IIB in 14 patients, and stage IV in 2 patients. In the patients with stage IIB disease, 11 patients had T4 disease, 2 patients had N3 disease, and 1 patient had T4N3 disease. Two patients with stage IV disease also had bone metastases. All lung cancer samples and paired nonmalignant lung tissues were frozen and stored at −80°C until analysis.

TRAP Assay

TRAP assays were performed as described by Kim and Piatyszek. Tissue samples of 50 to 100 mg were homogenized in 200 ml 3-(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS) lysis buffer (10 mM Tris-HCl, pH 7.5), 1 mM MgCl₂, 1 mM ethylene glycol-bis(b-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 10% glycerol, 5 mM beta-mercaptoethanol, and 0.1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF). After 30 minutes of incubation on ice, the lysates were centrifuged at 12,000g for 20 minutes at 4°C, and the supernate was rapidly frozen and stored at −80°C. The concentration of protein was measured using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), and an aliquot of extract containing 6 μg protein was used for each TRAP assay.

The TRAP assay was performed as follows. Each extract was assayed in 50 μl reaction mixture containing 50 μM each dNTP, 0.1 μg TS primer (5′-AATCCGTCGAGCA-GAGTT-3′), 0.5 μM T4 gene 32 protein (Boehringer Mannheim, Indianapolis, IN), 150 kBq [α³²P]dCTP, and 2 units of Taq DNA polymerase (Takara, Kyoto, Japan) in a 0.5-ml HotStart 50 tube (Molecular BioProducts, San Diego, CA) that contained 0.1 μg CX primer (5′-CCCTTAC-CCTTACCTTACCTTA-3′) sequestered at the bottom by a wax barrier. After 30 minutes of incubation at room temperature, the reaction mixture was then heated at 90°C for 3 minutes and was then subjected to 31 PCR cycles of 94°C for 40 seconds, 50°C for 40 seconds, and 72°C for 45 seconds. The PCR product was electrophoresed on a 10% acrylamide gel and then autoradiographed. The sample was classified as positive when the telomerase-specific 6 bp DNA ladder was observed after an overnight exposure. All telomerase-negative tumor samples were further checked by another TRAP round including a 150 bp internal telomerase assay standard to exclude the possibility of Taq DNA polymerase inhibitory activity in the tumor extracts.

Figure 1 shows examples of NSCLC and paired normal lung specimens with either positive or negative levels of telomerase activity.

Statistical Analysis

The associations between the telomerase activity and the clinicopathologic features were analyzed by chi square anal-
ysis. When the expected value was <5, we used Fisher’s exact test. Only age and tumor sizes were compared using the Mann-Whitney test. The survival curves of patients with telomerase-positive or -negative tumors were estimated by the Kaplan-Meier method, and differences between these survival curves were analyzed by the generalized Wilcoxon test. Univariate and multivariate survival analyses were performed using the Cox’s proportional hazards regression model. The statistical difference was considered significant at <0.05.

RESULTS

Telomerase Activity and Clinical Features

Telomerase activity was positive in 85 (82.5%) of 103 NSCLC tissue specimens analyzed and negative in 18 (17.5%). Telomerase activity was not detected in any normal lung tissue. Extracts from all telomerase-negative tumor samples gave a positive signal for the internal telomerase assay standard, excluding the possibility of Taq polymerase inhibition.

The relation between telomerase activity and clinical and pathologic features is summarized in Table 1. The mean age at surgery, sex, smoking, histologic type, and size of tumor extension were not different between the two groups. There were more cases of positive telomerase activity in the group with poorly differentiated tumors than in the group with well- and moderately differentiated tumors (p = 0.035). Telomerase activity was detected in 69 (79.3%) of 87 patients with stage IA to IIIA disease, whereas all 16 specimens from the patients with stage IIIB to IV disease demonstrated positive telomerase activity; the difference was statistically significant (p = 0.035).

Survival Analysis

The Kaplan-Meier survival curves in patients with NSCLC demonstrated that patients with telomerase-positive tumors survived for a significantly shorter period than those with telomerase-negative tumors (p = 0.0058) (Fig. 2). Because telomerase activity was detected in all patients with stage IIIB to IV disease, a survival analysis was made within the stage IA to IIIA group. The difference was significant regarding the survival curves between these two groups (p = 0.027). The difference was not statistically significant when a survival analysis was made within the stage IA and IB group (p = 0.201), but no patient with a telomerase-negative tumor has died up to now.

Table 2 shows a univariate survival analysis of the various prognostic factors using the Cox’s proportional hazards regression model. T stage, nodal status, differentiation of tumor, and telomerase activity were statistically significant prognostic factors (p < 0.05). A multivariate analysis with Cox’s proportional hazards regression model was performed to investigate the independent prognostic factors among these four factors (Table 3). As a result, only the T stage and telomerase activity were identified as independent prognostic factors (p = 0.0027 and 0.035, respectively).

DISCUSSION

In this study, telomerase activity was detected in 85 (82.5%) of 103 NSCLC tissue specimens analyzed using the
TRAP assay. In addition, all patients with advanced disease (stage IIIB to IV) demonstrated positive telomerase activity. Similar results have also been reported in other malignancies such as leukemia, malignant skin conditions, gastric cancer, and colon cancer. Telomerase activity was not detected in 10% to 20% of these malignant tumors when they were clinically diagnosed, and the rate of telomerase activation also increased with the progression of disease. In NSCLC, it is thought that most of the cells are not initially immortalized; therefore, the telomeres gradually approach critical shortening, and only a few cells can escape crisis (M2), acquire telomerase activity, and thus become immortal. It is thought that there are more telomerase-positive cells in advanced tumors than in early-stage tumors.

When we analyzed each T and N factor, telomerase activity was detected more frequently in T4 and N2-3 disease. In gastric cancer, Hiyama et al reported that the tumors with telomerase activity were significantly larger than those without telomerase activity. However, in this study no correlation was seen between telomerase activity and tumor size. It is thus thought that the size of the tumor in lung cancer may not necessarily represent the true aggressiveness of disease, because a small peripheral tumor sometimes metastasizes to mediastinal lymph nodes.

There were more cases of positive telomerase activity in the group with poorly differentiated tumors than in those with moderately and well-differentiated tumors. Similar results have been reported recently for hepatocellular carcinoma. These results may reflect the fact that patients with poorly differentiated lung cancers have a poorer prognosis.

Lung cancer is now thought to arise through accumulation of a number of genetic changes, such as mutations and deletions. Recent advances in molecular biology and genetics have created new diagnostic and treatment possibilities for clinical oncology. The potential prognostic implications of several biologic and molecular parameters, including oncogenes, such as K-ras mutation and c-erbB-2 overexpression, tumor suppressor genes, such as p53 abnormalities and cancer cell proliferative activity, have been reported in NSCLC. We also have reported the results of a systematic search for biologic and molecular prognostic markers such as p53 abnormalities, loss of heterozygosity at 3p and 9p, deletion of p16, and overexpression of vascular endothelial growth factor (VEGF) in NSCLC from our

<table>
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<th>Factors</th>
<th>Characteristics</th>
<th>Risk Ratio</th>
<th>95% CI</th>
<th>p Value</th>
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<td>T stage</td>
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<td>1.350–71.8</td>
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Table 3. MULTIVARIATE ANALYSIS OF PROGNOSTIC FACTORS

<table>
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<th>Characteristics</th>
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<tr>
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<td>Negative</td>
<td>8.616</td>
<td>1.168–63.57</td>
<td>0.0347</td>
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</table>

institution, our findings suggested that p53 abnormality and 3p loss of heterozygosity in adenocarcinoma, deletion of p16, and VEGF overexpression in squamous cell carcinoma of the lung may be prognostic factors. However, pathologic tumor staging is the most accurate factor currently available for predicting prognosis in patients who have undergone surgical resection.

One of the most interesting findings in this study was the prognostic impact of detection of telomerase activity in NSCLC. A survival analysis revealed a worse prognosis for patients with telomerase-positive tumors than those with telomerase-negative tumors. According to a multivariate analysis using Cox’s proportional hazards regression model, we identified telomerase activity as an independent prognostic factor (RR = 8.62, p = 0.035) of tumor stage. Previous studies have reported that telomerase activity is correlated with poor prognosis in neuroblastoma, gastric cancer, and leukemia. However, the influence of telomerase activity on prognosis has not yet been reported in NSCLC. If telomerase activity reflects the immortality of the cells, a good prognosis in patients with telomerase-negative tumors would thus be assumed as a matter of course. In all 18 patients with telomerase-negative NSCLC in this study, only 1 has died of cancer. In an additional patient, a brain metastasis developed 29 months after the lung surgery; however, the patient is still alive 21 months after resection of the brain metastasis.

In conclusion, telomerase activity was detected in 82.5% of NSCLC tissues, and it was detected more frequently in advanced disease than early-stage disease. Patients with telomerase-negative tumors are thus considered to have a better prognosis than those with telomerase-positive tumors. Telomerase activity in NSCLC may therefore be a useful predictor of prognosis, and it may be helpful in defining groups of patients who could benefit from surgical adjuvant treatments.

Acknowledgments

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References