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# Telomerase activity and serum levels of p53 protein as prognostic factors of survival in patients with advanced non-small cell lung cancer

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**Summary**
*Purpose:* Evaluation of relationships between survival time of patients with advanced, non-resectable non-small cell lung cancer (NSCLC) and telomerase activity in aspirates, collected from primary lung tumours, and serum p53 protein levels.
*Material and methods:* The study group consisted of 52 patients with advanced (stage IIIB and IV) non-small cell lung cancer. In all of them, transthoracic fine-needle biopsy (TFNB) of focal pulmonary lesion was performed. The aspirates were subjected to telomerase activity by the PCR–ELISA PLUS method and serum levels of p53 protein were determined by the ELISA method. Additionally, clinical advancement of cancer and the time period of survival were assessed in the studied group. Kaplan–Meyer method and Cox analysis were used for statistical evaluation of survival prognosis.
*Results:* Increased telomerase activity was observed in 42 (81%) of the patients with non-resectable non-small cell lung cancer. Elevated concentrations of serum p53 protein were found in 28 (54%) of the participants. The following death rates were noted during the entire study period: twenty-three (23) (62%), out of 37 patients with increased telomerase activity, 7 (47%), out of 15 without detectable telomerase activity in primary lung tumour, 16 (57%), out of 28 subjects with increased serum levels of p53 protein and 14 (58%), out of 24 with no increased serum levels of p53.

A significant relationship was observed in Cox hazard analysis between the time of survival and telomerase activity, while no such relationship was observed between the survival time period and serum p53 protein levels or sex, age, primary lung tumour size, lymph node status or development of distant metastases.

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*Conclusion:* Telomerase activity in advanced primary non-small cell lung cancer is a better predictor of patients' survival than serum levels of p53 protein. The assessment of telomerase activity supplements in the prognosis of survival in the course of non-resectable NSCLC.


## Introduction

Lung cancer is the most common malignant tumour all over the world and is the main cause of deaths related to neoplastic diseases. More than 80% of cases include non-small cell lung cancer (NSCLC).[1] In the majority of cases, NSCLC develops peripherally in the lung parenchyma and, therefore, transthoracic fine-needle biopsy (TFNB) is one of the principal methods of cytological diagnostics of such tumours. It is believed that in patients with NSCLC, the rates of activity of certain molecular markers may be helpful prognostic factors, regarding both the overall and free-of-recurrence survival periods.[2–5] Telomerase and p53 suppressor gene belong to the most important pathways of carcinogenesis in lung malignancies.[6–8]

Telomerase is a specific DNA polymerase, consisting of a protein component, comprising telomerase reverse transcriptase and telomerase-associated protein 1 and an RNA component, which is a template for the elongation of telomeric repeat sequences, located at the ends of DNA.[8,9] High-telomerase activity is characteristic, first of all, for cells of malignant tumours, being associated with their uncontrolled proliferation.[9] The p53 tumour suppressor gene is involved in the control of cell growth and programmed cell death.[7,8] The aim of the reported study was to assess the relationship between the survival of patients with advanced, non-resectable NSCLC and telomerase activity in aspirates, collected from primary lung tumours, as well as serum levels of p53 protein.

## Material and methods

Patients with non-resectable (stage IIIB and IV), non-small cell lung cancer have been enrolled to the study. In each patient, TNFB was done [Becton Dickinson needle 0.7 mm in diameter] under fluoroscopy control. After radiologically confirmed localisation of the needle into the tumour, an aspirate was collected and divided into two equal specimens for cytological and molecular examinations. The aspirates for cytological examination were smeared on defatted slides, fixed in 95% ethyl alcohol, and stained with eosin and haematoxylin. The specimens were separately evaluated by two pathologists and the final diagnosis was obtained by consensus between them. The pathologists were not informed about the level of telomerase activity in the specimens at any stage before the final diagnosis.

The aspirates for telomerase activity assessment were immediately placed in 1 ml probes and deeply frozen [at $-70\,^{\circ}C$]. PCR-ELISA$^{PLUS}$ (ROCHE Molecular Biochemicals, Mannheim, Germany) method was used for the assessment of telomerase activity, as described below:[10]

After defrosting, the aspirates were homogenized in 200 μl ice-cooled Lysis reagent. The lysate was centrifuged at $16\,000\times g$ for 20 min at $4\,^{\circ}C$. A volume of 175 μl of the supernatant was gently removed and its protein concentration measured with a Bio Rad Protein Assay Kit. Each supernatant was divided into two aliquots: one, inactivated at $85\,^{\circ}C$ for 10 min, was used as a negative control, while the other one was used to evaluate telomerase activity. A 3 μg volume of the protein extract was used for each assay. The telomerase activity assessment was done, according to the Telomeric Repeat Amplification Protocol [TRAP] method, consisting in amplification of telomeric sequences, added by telomerase to the 3′ end of biotin-labelled synthetic primer. Those elongation products, as well as the Internal standard [IS], which constituted a positive control, included in the same reaction vessel, were amplified, using appropriate primers. The PCR products were split into two aliquots, denaturated and hybridized to digoxigenin-labelled probes, specific for the telomeric repeats and for the IS, respectively. The resulting products were immobilized via the biotin label to a streptavidin-coated micro titre plate. The immobilized amplicons were then detected with an anti-digoxigenin antibody, conjugated to horseradish peroxidase and sensitive peroxidase substrate. The absorbance of the samples was measured, using an ELISA reader with 450 nm wavelength. The samples were considered as telomerase positive if the difference in absorbance was higher than the background activity. As proposed by the manufacturer, relative telomerase activity units (RTA) were used for the quantitative assessment of telomerase activity.[10]

Seven ml of venous blood was collected from each participant. The photometric one-step-enzyme-immunoassay method for the quantification of circulating p53 [ROCHE Molecular Biochemicals, Mannheim, Germany] was used.[11] The assay is based on the quantitative ''sandwich ELISA'' principle. P53 activity was measured twice in each sample, and its mean value was used for further analysis. The samples with signal twice higher than the zero-standard signal were considered as positive. Serum p53 concentrations were expressed in pg/ml.

The survival of patients was evaluated with respect to telomerase activity in TFNB aspirates and serum p53 levels by the Kaplan–Meier method. Patients with the status which could have a significant influence on their survival, i.e., complete respiratory failure or severe blood circulation failure, haemoglobin concentration below 11 g/dl, hypoalbuminemia ($<3$ g/dl), serum lactate dehydrogenase concentration above 150 U/l, platelet count above 600 g/l, hypercalcemia ($>2.75$ mmol/l) or bad general status ($\geq 3$ at Zubrod's scale) were excluded.[12–17] Patients with malignant neoplastic diseases during previous 5 years, as well as patients with NSCLC, recognised before TFNB were not included, either. Deaths because of cancer or its recurrence during the study period were qualified as complete observation and analyzed separately. Tumour size, lymph node invasion and distant metastases were determined in all the cancer subjects according to International System for Staging Lung Cancer.[1,18] In patients who undergone

**Table 1** Overall survival of patients with non-resectable NSCLC in relation to telomerase activity and p53 protein expression.

| | Number of patients | Observation period | |
|---|---|---|---|
| | | Mean overall survival (95% CI) (days) | Number of complete observations (deaths), *n* (%) |
| Telomerase (−) p53 (−) | 8 | 330.6 (244.2—417.0) | 4 out of 8 (50.0%) |
| Telomerase (−) p53 (+) | 7 | 347.7 (193.3—502.2) | 3 out of 7 (43.0%) |
| Telomerase (+) p53 (−) | 16 | 248.4 (162.6—334.3) | 10 out of 16 (62.5%) |
| Telomerase (+) p53 (+) | 21 | 253.1 (187.1—319.2) | 13 out of 21 (62%) |
| Overall | 52 | 276.4 (234.8—317.9) | 30 out of 52 (58.0%) |

open lung biopsy, the final stage of cancer stage was determined by a combination of pathomorphological and clinical findings, according to the current TNM classification for lung cancer staging.[1,18]

Relationships were assessed between patient survival on one hand and patient age and sex, telomerase activity in primary tumour, serum p53 protein levels and the clinical stage of NSCLC on the other, employing the Cox hazard analysis. The 95% confidence interval (CI) was set and $p$ value $\leq 0.05$ was considered as statistically significant. All of the statistical analyses were performed with the STATISTICA PL 7.0 program (serial No. AAAP510C860213FA). The study protocol was approved by the Ethics Committee of the Military Medical Chamber.

## Results

The analyzed group comprised 52 patients [15 women and 37 men, mean age — 66.4 (95 CI: 63.1—69.8) years] with newly diagnosed, non-small cell lung cancer, including 28 patients with stage IIIB and 24 patients with stage IV of the disease. Cytological diagnosis of lung tumour was established from TFNB results in 45 (86.5%) patients. In four cases, histological diagnosis of tumour was done after open lung biopsy and in three cases, after bronchial brushing during bronchoscopy. Detectable telomerase activity was found in 42 (81%) aspirates, obtained during the TFNB. Detectable telomerase activity was found in 29 (80.5%), out of 36 histologically unspecified non-small cell lung cancers, 4, out of 6 (66%) squamous cell cancers and in 9, out of 10 adenocarcinomas. Elevated serum p53 protein concentrations were observed in 28 (54%), out of 52 patients, including 23 cases of unspecified NSCLC, 1 case of squamous cell carcinoma and 4 cases of adenocarcinoma. All the patients were treated by oncologists, according to the evidence-based medicine principles. Forty patients were treated only with chemotherapy and/or radiotherapy. The best supportive therapy was conducted in the remaining twelve patients (3 patients didn't give consent for more aggressive treatment, 2 were older than 80, 2 had massive pleural effusions, and last 5 patients had severe chronic obstructive pulmonary disease).

During the study period, 23 (62%) patients died, out of 37 patients with detectable telomerase activity, 7 (47%), out of 15 without detectable activity of telomerase in primary tumour, 16 (57%), out of 28 subjects with high serum levels of p53 protein and 14 (58%), out of 24 without increased serum p53 levels (Table 1). The mean periods of survival, according to telomerase activity and p53 protein concentrations, are presented in Table 1.

It was shown that the presence of telomerase activity in aspirates derived from primary lung tumour, opposite to increased levels of p53 protein in serum, is a significant predictor of survival in patients with advanced, non-operable NSCLC (Figs. 1 and 2). Neither were there any significant differences in the probability of survival among 4 subgroups of patients, divided with respect to increased serum p53 protein levels and/or telomerase activity in cancer tissue (Fig. 3).

A statistically significant relationship was observed in Cox hazard analysis between the time of survival and the quantitatively measured telomerase activity levels (in RTA units). There was no such relationship though between the survival period on the one hand and serum levels of p53 protein, sex, age, primary lung tumour size (T feature), lymph node occupation or the presence of distant metastases on the other (Table 2).

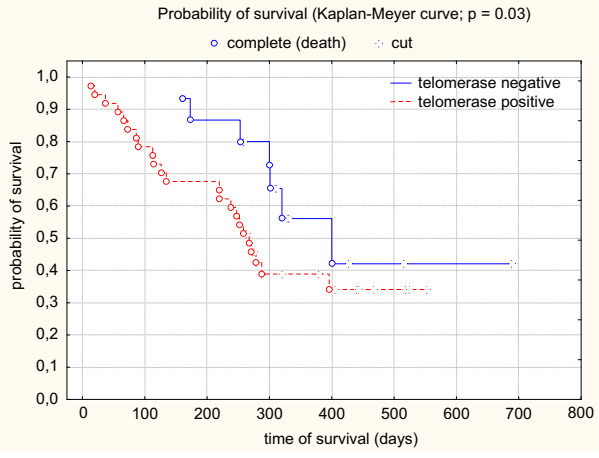


**Figure 1** Probability of survival with respect to telomerase activity in lung tumour, observed in the studied group.

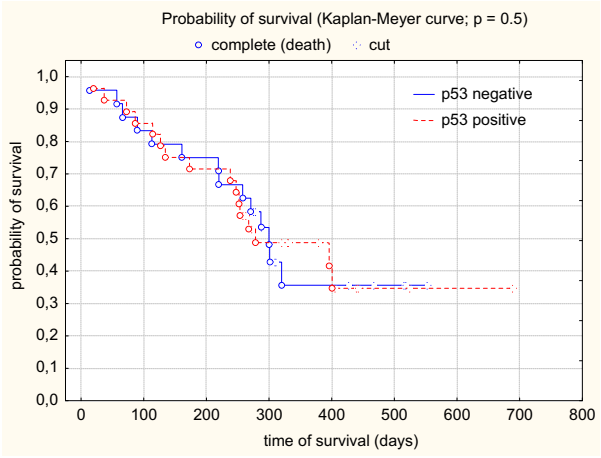


**Figure 2** Probability of survival with respect to increased serum levels of p53 protein in the studied group during observation period.

## Discussion

In the last decade, numerous studies have shown highly increased telomerase activity in more than 80% of different carcinomas, while either no or very low expression in normal tissues.[3–5,9,19–22] In patients with lung carcinoma, high-telomerase activity has been observed in sputum, bronchial washing and brushing specimens obtained during bronchofibroscopy.[2,22] In our previous studies we proved that assessment of telomerase activity in lung infiltrations is a useful supplemental procedure in differentiation between malignant and benign tumours.[23] It is thought then that telomerase, as an enzyme responsible for unlimited replication of genetic material inside cancer cells and incomplete cell divisions, could be a molecular prognostic factor. Fujita et al.[24] observed that strong and moderate expression of human telomerase reverse transcriptase mRNA (hTERT) in resected NSCLC was a prognostic factor of survival with predictive power similar to that of lymph node

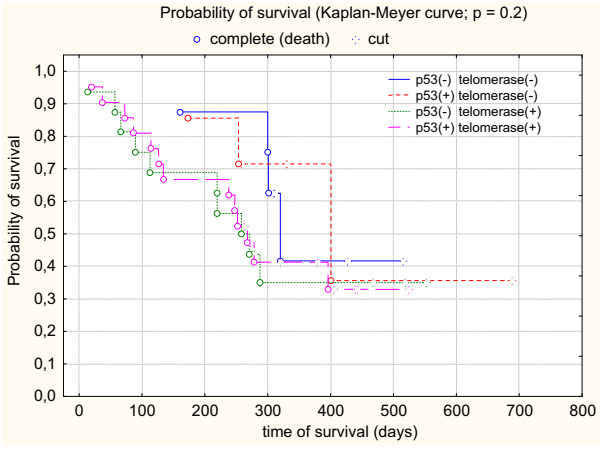


**Figure 3** Probability of survival with respect to increased serum levels of p53 protein and telomerase activity in lung tumour in the studied group during observation period.

**Table 2** Cox hazard analysis of the influence of selected factors on the survival in patients with advanced NSCLC ($p = 0.046$*).

| | *p* |
|---|---|
| Relative telomerase activity<br>Mean (95%CI)<br>34.7 (21.6—47.9) units | 0.007* |
| Serum level of p53<br>Mean (95%CI)<br>153.0 (97.7—208.4) pg/ml | 0.16 |
| Age<br>Mean (95% CI)<br>66.4 (63.1—69.8) years | 0.5 |
| Sex (number of cases)<br>Female — 15<br>Male — 37 | 0.6 |
| *T* (number of cases)<br>$T_1$ — 5<br>$T_2$ — 21<br>$T_3$ — 10<br>$T_4$ — 16 | 0.09 |
| *N* (number of cases)<br>$N_0$ — 10<br>$N_1$ — 14<br>$N_2$ — 13<br>$N_3$ — 15 | 0.1 |
| *M* (number of cases)<br>$M_0$ — 28<br>$M_1$ — 24 | 0.9 |

* Significant $p$ value $\leq 0.05$.

status, pathomorphological TNM stage and the age of patients. Some authors did not find any association between clinicopathological features of lung cancer and hTERT status, however, they observed longer survival periods of patients without telomerase activity in primary tumours.[25] So far, telomerase prognostic value in lung cancer has been studied on specimens derived from less advanced, surgically resectable tumours.[4,24,26,27] Moreover in normal cells and tissues, the levels of wild type of p53 are very low, whereas in malignant tissues and cancer lines, a mutated p53 polypeptide is often detectable in high concentrations.[28–30,33] It is believed that more advanced stages of lung cancer could be associated with higher p53 activity in blood serum.[29] So far, several studies have been conducted in which the presence of p53 antibodies, the levels of p53 protein or expression of p53 suppressor gene correlated to various clinical parameters in patients with cancer.[29,31–36] Most of all, the studies on the prognostic value of p53 in lung cancer were conducted in patients who had undergone surgical treatment because of non-small cell lung cancer.[31,32,35] There are only a few publications on the prognostic role of p53 in patients with advanced lung cancer.[29]

In this study, we tried to assess if telomerase activity in aspirates, derived from primary lung tumours, and serum levels of p53 protein could be valuable prognostic factors in

patients with advanced non-small cell lung cancer (stages IIIB plus IV). Following Fujita et al.[24] we selected some variables for analysis of survival. It was revealed that only telomerase activity correlated significantly with obtained lifetime. There was no significant relationship between the survival period and serum levels of p53 protein, the age and sex of patients, primary tumour size (T), lymph node status (N) or the presence of distant metastases in the studied group (Table 2). We may conclude then that telomerase activity in advanced primary non-small cell lung cancer is a better predictor of patient survival than serum levels of p53 protein. The assessment of telomerase activity supplements the prognosis of survival in the course of advanced, non-resectable NSCLC.

## Conflict of interest statement

There is no any conflict of interest.

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