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Telomerase activity in transthoracic fine-needle biopsy aspirates as a marker of peripheral lung cancer

Running title: Telomerase activity in fine-needle biopsy aspirates
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ABSTRACT

The Aim of the Study: Evaluation of usefulness of telomerase activity assay in transthoracic fine-needle biopsy [TFNB] aspirates collected from peripheral tumours of the lung in predicting of the malignant aetiology of lung infiltrations.

Material and Methods: The study group consisted of one hundred patients with a peripheral infiltration of the lung. All of them had TFNB of the focal lesion performed. The aspirates were subjected to the standard cytological evaluation. Telomerase activity in the specimens was determined with the PCR-ELISA PLUS method. The sensitivity, specificity, accuracy and predictive value of TFNB were calculated for the cytological examination of aspirates alone and the cytological examination with additional telomerase activity assessment.

Results: Lung cancer was newly diagnosed in 84 subjects, and benign peripheral lesions were found in 16 subjects. During the first TFNB lung cancer was recognized in 56 [66.7%] cancer cases, while increased telomerase activity was found in 61 [72.6%] cancer aspirates. Nobody with a benign infiltration had a false positive result of the cytological examination, but in 1 case [6.25%] increased telomerase activity was observed. The diagnostic sensitivity, accuracy and negative predictive value of the combination of cytological examination and telomerase activity assay in TFNB specimens were significantly higher than those of the cytological examination alone [89.3% vs. 66.7%, p=0.0004; 90% vs. 72.%, p=0.001; 62.5% vs. 36.4%, p=0.039], however performance of both examinations was associated with a lower specificity of TFNB [96.9% vs. 100%, p=0.002].

Conclusion: Detection of telomerase activity in aspirates taken during TFNB of a peripheral lung infiltration should be considered as a warning of the risk of malignancy in case of false-negative results of cytology.
Introduction
Suspicious lung infiltrations, including malignant tumours, are frequently located peripherally in the lung parenchyma and transthoracic fine-needle biopsy (TFNB) appears to be a useful method of cytological diagnosis of such lesions [1-3]. However, TFNB has drawbacks which are a small amount of cells obtained during the procedure and quite high risks of false negative results and complications, such as pneumothorax [4-7]. The absence of neoplastic cells in smears collected during fine-needle biopsy is not sufficient evidence of a benign character of lung lesions, and it cannot be the grounds to abandon further diagnosis of the lung infiltration. Additional assessment of specific markers of malignant disorders in TFNB aspirates could improve the sensitivity of TFNB and diminish the risk that malignant lung cancer will remain unrecognized. Telomerase, a ribonucleic enzyme responsible for uncontrolled proliferation of cancer cells, is one of highly specific molecular markers of malignant diseases [8]. It is believed that increased telomerase activity could be a helpful indicator of the malignant aetiology of the tumour and a prognostic factor of cancer development and patient survival [8, 9]. In the study, it was assessed how evaluation of telomerase activity in aspirates from peripheral lung infiltrations influences the diagnostic value of transthoracic fine needle biopsy.

Material and methods
The study involved 100 patients with a peripheral (located beyond the field of vision of a bronchoscope) focal infiltration of the lung, who were qualified for transthoracic fine-needle biopsy. All the patients had CT of the lung done. Indications for TFNB included (1) solid tumours with irregular contours, noncalcified or with peripheral, disseminated or spotted calcifications; (2) perimediastinal location of lesions (which could suggest small cell lung cancer); (3) enlargement of mediastinal lymph nodes (patients candidates for preoperative therapy in case of lung cancer); (4) distant extrapulmonary changes (i.e. in brain, bones or liver) which might appear to be metastatic foci of primary lung tumour.

TNFB was performed [Becton Dickinson needle 0.7 mm in diameter] under fluoroscopy control. After radiologically proven placement of the needle in the tumour, an aspirate was taken and divided in two equal specimens for cytological and molecular examinations. The aspirates for the cytological examination were smeared on defatted slides fixed in 95% ethyl alcohol, and stained with eosin and haematoxylin. The specimens were evaluated by two pathologists separately and the final diagnosis was established by consensus between them. The pathologists were not informed about the level of telomerase activity in the specimens at any moment before they reached the final diagnosis.

The aspirates for the telomerase activity assessment were immediately placed in 1 ml probes and deeply frozen [at –70°C]. The 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 x g for 20 min at 4°C. 175 µl of the supernatant was gently removed and its protein concentration was measured with the Bio Rad Protein Assay Kit. Each supernatant was divided into two aliquots. One, inactivated at 85°C for 10 min, was used as a negative control, while the other one was used to evaluate telomerase activity. 3 µg of 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. These elongation products, as well as the Internal standard [IS], which constituted a positive control, included in the same reaction vessel, were amplified using the appropriate primers. The PCR products were split into two aliquots, denatured 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. Immobilized amplicons were then detected with an anti-digoxigenin antibody that is conjugated to horseradish peroxidase and the sensitive peroxidase substrate. The absorbance of the samples was measured using an ELISA reader with 450 nm of wavelength. The samples were considered as telomerase positive if the difference in absorbance was higher than the background activity.

TFNB sensitivity, specificity, accuracy and predictive value in diagnosis of malignant and benign aetiologies of lung infiltrations were assessed. The above-mentioned parameters were calculated for the cytological examination alone and for the cytology with telomerase activity assessment in the aspirates. Aspirates from malignant tumours with cancer cells and/or the presence of telomerase activity were considered as true positive, aspirates from benign infiltrations of the lung without cancer cells and/or telomerase activity were regarded as true negative. Aspirates taken from malignant tumours without cancer cells and/or telomerase activity were considered as false negative, and aspirates from benign infiltrations with recognized cancer cells in the aspirates or increased telomerase activity were regarded as false positive. Differences in the sensitivity, specificity, accuracy, positive and negative predictive values of TNFB were evaluated with the chi-square and Fisher tests. The concordance between the presence or absence of telomerase activity and the presence or absence of cancer cells in aspirates was calculated. The Mann-Whitney test was used to compare the mean size of suspected infiltrations in patients in whom telomerase activity results agreed with cytological findings and patients in whom these results were not consistent. The confidence interval [CI] was set at 95%.

The study protocol was approved by the Ethics Committee of the Military Medical Chamber [n° 59/2002]. The study was financially supported by the Polish Ministry of Science.

Results
The mean size of suspicious lesions was 2.4 [CI: 2.2-2.5] cm. Lung cancer was proved in 84 out of 100 participants, while a benign peripheral infiltration was finally recognized in 16 subjects.

After the cytological examination of aspirates collected during the first TFNB, cancer was recognized in 56 [66.7%] patients, including 52 [61.9%] cases of non-small cell lung cancer and 4 [4.8%] of small cell lung cancer. Moderate atypia was observed in 5 [5.9%] cases and smears of 23 [27.4%] cancer subjects were non-diagnostic [i.e. contained necrotic, purulent or epithelial cells or cells with mild atypia or other non-specific blood cells]. Of 28 patients without the diagnosis of cancer after the evaluation of smears from the first TFNB, 10 [12%] subjects had NSCLC recognized after subsequent aspirations (performed within 7 days after the first TFNB with a 0.9 mm gauge needle), 15 [17.9%] had the histological diagnosis of NSCLC established after open lung biopsy and 2 [2.4%] after another procedure [1 - resection of brain metastases, 1 - bronchial brushing during bronchofibroscopy], and 1 had SCLC diagnosed after surgical biopsy. Increased telomerase activity was observed in 61 [72.6%] of 84 aspirates from peripheral malignant tumours of the lung, including 19 cases without cancer cells in smears. In only 9 cases were neither cancer cells nor
telomerase activity found in aspirates derived from peripheral malignant tumours of the lung [Table 1].

Table 1. Telomerase activity and cytological examination results in lung cancer group

<table>
<thead>
<tr>
<th>Telomerase activity</th>
<th>NSCLC</th>
<th>SCLC</th>
<th>Atypical cells</th>
<th>Non-diagnostic*</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>14 [26.9%]</td>
<td>0 [0%]</td>
<td>1 [25%]</td>
<td>8 [34.8%]</td>
<td>23 [27.4%]</td>
</tr>
<tr>
<td>positive</td>
<td>38 [73.1%]</td>
<td>4 [100%]</td>
<td>4 [75%]</td>
<td>15 [65.2%]</td>
<td>61 [72.6%]</td>
</tr>
</tbody>
</table>

* [i.e. mild atypia, necrotic, purulent, bronchial epithelial cells, granulocytes, erythrocytes, etc.]

Of the 16 subjects with benign focal infiltrations, 8 had non-specific inflammatory infiltrations, 4 had tuberculosis, 3 had active tuberculosis and 1 had hamartoma. In 1 case of inflammation, atypia was observed. There were no false positive cytological results in this group of patients, but increased telomerase activity was observed in 1 case [6.25%] of non-specific inflammation of the lung (with atypical cells). The concordance between the presence or absence of telomerase activity and the presence or absence of cancer cells in aspirates was 66%.

There were no significant differences in the mean size of suspected infiltrations between the patients in whom telomerase activity results agreed with cytological findings and the patients in whom these results were not consistent – 2.4 [CI 2.3-2.6] cm and 2.3 [2.1-2.5] cm, respectively (p = 0.48).

It was revealed that the additional assessment of telomerase activity in TFNB aspirates from peripheral lung infiltrations significantly improved the sensitivity, accuracy and negative predictive value of fine-needle biopsy, however it was associated with decrease of TFNB specificity [Table 2].

Table 2. Diagnostic value of TFNB in lung cancer

<table>
<thead>
<tr>
<th></th>
<th>cytological examination alone</th>
<th>telomerase activity assessment alone</th>
<th>cytological examination plus telomerase activity assessment</th>
<th>p [chi² test]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>sensitivity %</td>
<td>66.7% [CI: 56.1%-75.8%]</td>
<td>72.6% [CI: 62.3%-81.0%]</td>
<td>89.3% [CI: 80.9%-94.3%]</td>
<td>p = 0.0004</td>
</tr>
<tr>
<td>specificity %</td>
<td>100% [CI: 80.6%-100%]</td>
<td>93.8% [CI: 71.7%-98.9%]</td>
<td>96.9% [CI: 84.3%-99.5%]</td>
<td>p = 0.002*</td>
</tr>
<tr>
<td>accuracy %</td>
<td>72.0% [CI: 62.5%-79.9%]</td>
<td>76.0% [CI: 66.8%-83.3%]</td>
<td>90% [CI: 82.6%-94.5%]</td>
<td>p = 0.001</td>
</tr>
<tr>
<td>negative predictive value %</td>
<td>36.4% [CI: 23.8%-51.1%]</td>
<td>39.5% [CI: 25.6%-55.3%]</td>
<td>62.5% [CI: 42.7%-78.8%]</td>
<td>p = 0.039</td>
</tr>
<tr>
<td>positive predictive value %</td>
<td>100% [CI: 93.6%-100%]</td>
<td>98.4% [CI: 91.4%-99.7%]</td>
<td>98.8% [CI: 93.6%-99.8%]</td>
<td>p = 0.4</td>
</tr>
</tbody>
</table>

*Fisher test

Discussion
Owing to high accessibility of imaging techniques, peripheral focal lesions of the lung with unknown aetiology are often observed [11]. In order to determine the malignant or benign character of those lesions, transthoracic fine-needle biopsy is performed [2, 5]. However, TFNB is an oligo-cell technique burdened with limited sensitivity [4, 7, 13].

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In addition, it is an invasive method with the risk of serious complications, such as pneumothorax, which in unfavourable conditions occurs even in a half of cases [5].

Telomerase activity assessment in TFNB aspirates could be a helpful tool in differential diagnosis of malignant and benign focal lesions of the lung. Although sporadic cases of high telomerase expression in a fatal course of pneumonia and cystic fibrosis have been described [14], high telomerase activity is characteristic first of all of cells of malignant tumours and is responsible for their uncontrolled proliferation [8]. In healthy mature organisms, telomerase activity is detectable in fast-dividing cells, such as germ cells, epithelial cells, lymphocytes or activated fibroblasts [8, 15]. However, it is definitely lower than in malignant neoplastic tissues. For example, it has been shown that the activity of telomerase in oesophageal cancer cells is 600 fold higher than in diploid fibroblasts [16].

In the study, increased telomerase activity was found in 72.6% of aspirates taken from malignant tumours of the lung, including 19 cases without the presence of cancer cells in smears. The assessment of telomerase activity, performed in addition to the cytological examination of aspirates, improves significantly the sensitivity, accuracy and negative predictive value of TFNB, however it is associated with small but statistically significant worsening of the specificity of fine-needle biopsy, due to the presence of telomerase activity in 1 benign case. Of course, it cannot be excluded that dividing of aspirates into two equal parts, necessary to perform telomerase assay, could slightly decrease the cytological sensitivity of TFNB, however the 66.7% sensitivity of the first fine-needle biopsy, achieved in the study, is comparable with the results of other authors [4, 12, 13]. Studies suggesting an even higher than the 90% sensitivity of biopsy are also described, however such excellent results may be reached only in tumour located directly by the thoracic wall, and those biopsies are often associated with a high risk of pneumothorax or even false positive cytological results [5].

Improvement of the negative predictive value from 36.4% to 62.5% means that the absence of cancer cells and lack of telomerase activity in the aspirate from the peripheral infiltration of the lung increases the probability of a benign character of the pulmonary lesion almost two times.

So far, only in case of breast tumours a significant improvement of fine-needle biopsy sensitivity was observed thanks to the additional assessment of telomerase activity in aspirates [17, 18]. An additional advantage of the evaluation of telomerase activity in tumours would be the possibility of identifying patients with a potentially unfavourable prognosis, as the high level of telomerase activity is an unfavourable prognostic factor in lung carcinoma, not related to tumour histological type, cancer clinical stage, age, sex and smoking habit [9, 19, 20].

In summary, detection of telomerase activity in oligo-cell aspirates from peripheral tumours of the lung could be a helpful warning of a malignant origin of the lung infiltration when no cancer cells are found in those aspirates.

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