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Comparison of Telomerase Activity in Malignant and Benign Pleural Effusions

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ABSTRACT

Background: *Despite the advances in diagnosis and treatment of lung cancer, its survival rate has only improved in those with early stages of disease. Telomerase is a tumor marker that has been focused on recently as a novel tool for early diagnosis of lung cancer. This study aimed to compare telomerase activity in cases with malignant and benign pleural effusions.*

Materials and Methods: *Telomerase activity was assessed in 28 consecutive cases of pleural effusions (19 cases with malignant and 9 cases with benign histopathologic diagnosis) with telomeric repeat amplification protocol (TRAP) between Apr. 2006 and Sep.2007. Data analysis was performed by using Chi-square test and t-test.*

Results: *Twenty (71.4%) out of 28 cases with pleural effusions were positive for telomerase activity. Telomerase activity was positive in all 19 malignant effusions, while only one case with effusion due to a benign condition (TB) had positive telomerase activity ($p<0.0001$). The sensitivity, specificity and diagnostic accuracy of telomerase activity for detecting malignant pleural effusions were 100%, 88.9% and 96.4%, respectively. Positive and negative predictive values of telomerase activity were 95% and 100%, respectively. Mean relative telomerase activity was not significantly different in malignant and benign effusions ($24.3\pm5.2\%$ vs. 15.05%; $p>0.05$)*

Conclusion: *Telomerase activity is a highly sensitive and specific diagnostic biomarker for malignancy and may be used as an adjunct to other diagnostic tools such as cytology for malignant pleural effusions.* ***(Tanaffos 2009; 8(2): 17-23)***

Key words: *Pleural effusion, Lung cancer, Telomerase*

INTRODUCTION

Lung cancer is a major cause of cancer-related mortality in the world. Although significant advances have been achieved in diagnosis and treatment of lung cancer, survival of patients has only been improved in those diagnosed in early stages of disease. Therefore, studies have focused on new methods for early detection of lung cancer. These efforts have resulted in discovery of various oncogenes and tumor suppression genes and molecules. But, most of these molecular markers have no clinical implications (1). Telomerase is one

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of these markers that appears to play a role in clinical settings.

Telomerase is a specialized reverse transcriptase that synthesizes telomeric repeats at the ends of the chromosomes (2). It seems that telomerase activity is limited to germinal and proliferating cells in some tissues with self- renewal capability (3).It has been illustrated that telomerase is permanently activated in some malignancies such as lung, prostate, liver and colon cancers. Total prevalence of telomerase activity was assessed in 85% of more than 3000 human tumor samples by using telomeric repeat amplification protocol (TRAP) assay indicating that telomerase is a universal biomarker for cancers (4,5).

Although histopathologic examination is the gold standard for lung cancer diagnosis, it requires high experience and adequate specimen. Furthermore, there is no agreement about definite pathological criteria in some tumors. With regard to the superiority of less invasive and more cost-effective methods for diagnosis of the disease, telomerase activity can be considered as a useful diagnostic marker for tumors (1,6,7).

The sensitivity and specificity of this tumor marker in malignant bronchoalveolar lavages and pleural effusions were reported to be 82.5% and 80.4%, respectively. Its sensitivity for benign pulmonary diseases, such as TB has been 19.6% (8, 9). All cell lines of human lung cancer and most lung cancer tissues (87%) showed telomerase activity, but studies failed to show any telomerase activity in normal tissues. Thus, telomerase activity may play a role in both development and progression of lung cancer (10, 11).

However, most studies have postponed the definite conclusion to further investigations. In this study, we evaluated the telomerase activity in cases with malignant and benign pleural effusions.

MATERIALS AND METHODS

Patients and samples

Twenty-eight consecutive patients with malignant (n=19) and benign (n=9) pleural effusions who underwent diagnostic thoracentesis in Rasoul-e-Akram Hospital in Tehran between April 2006 and September 2007 were selected. There were 21 males and 7 females with the mean age of 59.0±13.1 yrs (range 42–80 yrs). Diagnosis was made based on cytological and/or histopathological examinations for malignant diseases (11 primary lung cancers [58%], 4 metastatic cancers [21%] and 4 lymphomas [21%]) and clinical, radiographic and case-specific evaluations, such as cultures, perfusion-ventilation scan, histologic examinations and etc. for benign conditions (Table1).

Determination of telomerase activity

Telomerase activity assays and other examinations (especially histological examinations) were performed independently in a blinded manner. Telomerase activity was determined by polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) assay. In this photometric enzyme immunoassay, Telo TAGGG Telomerase PCR ELISA^{plus} (Roche kit) with synthetic P1-TS primer(3'TTAGGG) was used for determination of telomerase activity and the results were reported as relative telomerase activity (RTA).Following steps were performed on the sample derived from pleural fluids:

1. The fluid sample was transferred to the laboratory within 30 minutes in cold condition and cold PBS was added to the resultant cellular deposits after centrifugation. The sample was washed. The upper fluid was removed and 0.5 $_{ml}$ of cold PBS was added again. Then the cell count was conducted.
2. Samples were transferred to a -10°C freezer.
3. While performing the test, 2.9×10^5 cells were removed from the tubes according to the kit

instructions and washed once or cold PBS was added as mentioned earlier.

4. 200 ml of lysing solution was added to the cell deposit. It is important to note that two tubes were prepared in this manner, one of which was for negative control.
5. The tubes were incubated with ice for 30 minutes and then centrifuged for 20 minutes in 12,000 rpm at 9°C. The upper fluid (about 160_{pcl}) was gently transferred to another tube and one of the supernatant tubes was heated to 85°C for 10 minutes in order to inactivate the telomerase enzyme (negative control tube). PCR was conducted for each negative control and test sample tube so that the telomerase enzyme would produce and amplify the product or the same telomeric sequence if activated.
6. After PCR, two new tubes were provided for each test tube and one for negative control tubes. Then denaturizing and hybridizing materials were added, respectively.
7. 100 pcl of each tube was transferred to a microplate and re-incubated in 37°C for one hour, the hybridization product was emptied, and the plate was washed three times.
8. 100 pcl of anti-Digoxigenin solution was added which is marked by HRP enzyme and then incubated for half an hour at 15-25° C while homogenizing.
9. Next steps after incubation and removal of upper fluid included: 5 times of washing with the kit washing solution, adding TBM substrate, incubating for 10 minutes in 15-25° C, adding 100^{pcl} of stopper solution and finally, reciting the light absorption of microplate wells in wave length of 450 nm.
10. RTA was calculated by using the below-mentioned formula:

$$\textbf{RTA}= \frac{(A_S - A_{SO}) / A_{S,SI}}{(A_{TS8} - A_{TS8,0}) / A_{TS8,IS}} \times 100$$

A_S: absorbance of sample

$A_{S,0}$: absorbance of heat- or RNase- treated sample

$A_{S,SI}$: absorbance of internal standard (IS) of the sample

A_{TS8}: absorbance of control template (TS8)

$A_{TS8,0}$: absorbance of lysis buffer

$A_{TS8,IS}$: absorbance of Internal Standard (IS) of control template (TS8)

Statistical Analysis

Results were expressed in terms of frequency, percentage, and mean ± SD. Comparison of diagnostic values of telomerase activity and relative telomerase activity (RTA) in malignant and benign pleural effusions was performed by using Chi-square test and t-test, respectively. A p-value of <0.05 was considered statistically significant.

RESULTS

There were19 cases of malignant and 9 cases of benign pleural effusions (Table1). The mean ages of patients with malignant and benign pleural effusions were not significantly different (62±13.5 vs. 56±11.7, respectively; P>0.05)

Table1. Frequency of diseases associated with pleural effusion

Disease	Frequency
Malignant	
Lung cancer	11 (58%)
Metastatic cancer	4 (21%)
Lymphoma	4 (21%)
Total	19 (100%)
Benign	
Tuberculosis	2 (22.2%)
Antiphospholipid synd.	1 (11.1%)
Chronic heart failure	1 (11.1%)
Chronic renal failure	1 (11.1%)
Pneumonia	1 (11.1%)
Pulmonary thromboembolism	1 (11.1%)
Cirrhosis	1 (11.1%)
SVC syndrome	1 (11.1%)
Total	9 (100%)

The benign group included 4 (44.4%) females and 5 (55.6%) males, while the malignant group included 3 (15.8%) females and 16 (84.2%) males (P<0.001).

Twenty (71.4%) out of 28 pleural effusion samples showed positive telomerase activity.

All malignant pleural effusions showed positive telomerase activity, whereas, only one (11.1%) of 9 benign pleural effusions was positive for telomerase activity, which was related to a patient with TB. Comparison of telomerase activity results in both groups is shown in Table 2.

Table 2. Comparison of telomerase activity (TA) in malignant and benign pleural effusions

TA \ Disease	Malignant	Benign	Total
+	19	1	20
-	0	8	8
Total	19	9	28

The sensitivity and specifity of telomerase activity for detecting malignant pleural effusions was 100%, and 88.9%, respectively.

Telomerase activity had a positive predictive value of 95% and a negative predictive value of 100%.

Diagnostic accuracy of telomerase activity was 96.4%.

There was no significant difference in mean relative telomerase activity (RTA) between the malignant and benign pleural effusions (24.3±5.2% vs. 15.05%, respectively; P>0.05).

There was no significant difference in mean relative telomerase activity (RTA) between men and women with positive telomerase activity (24.56 ±4.9% vs. 19.6 ± 7.6 %, respectively; P>0.05).

DISCUSSION

Studies which evaluated diagnostic value of telomerase activity in malignant bronchoalveolar lavages and pleural effusions reported that the sensitivity and specificity of this tumor marker were 82.5% and 80.4%, respectively. Its sensitivity for TB was 19.6% (8.9). Moreover, all cell lines of human lung cancer and most lung cancer tissues (87%) showed telomerase activity, but studies failed to show any telomerase activity in normal tissues. Thus, telomerase activity may play a role in both development and progression of lung cancer (10, 11).

Previous studies reported various diagnostic values for telomerase activity in lung cancer, and some questioned the issue (12). Altogether, most studies postpone the definite conclusion to further investigations.

However, male/female ratio in the malignant group was significantly higher than in the benign group (5.3 vs.1.2, respectively; p<0.001). This is due to the higher prevalence of lung cancer in men and limited number of patients in our setting.

Our findings indicated that the frequency of telomerase activity in malignant pleural effusions was significantly higher than in benign effusions. Similar results were reported by previous studies, but few studies have shown telomerase activity in all lung cancers. For example, Dikmen et al. (2003) reported positive telomerase activity test in 82.5% of malignant pleural effusions and 19.6% of benign effusions (8). In a study conducted by Lee et al., 78% of lung cancer tissue samples showed telomerase activity (10). Yang and Xie (2001) showed that 5.7% of the benign pleural effusions expressed a weak telomerase activity, whereas 90% of the malignant effusions expressed a significant telomerase activity (13). The modest telomerase activity in our literature review was related to Dejmek et al. study conducted in a university hospital in Sweden on over 16 pleural effusions; in his study, 67% of the malignant effusions(n=9) expressed significant telomerase activity using TRAP method (14).

The sensitivity of telomerase activity for malignant pleural effusions was 100% in our study. This finding indicated that telomerase activity assay can detect all patients with lung cancer. Previous studies have shown similar results, but the sensitivity of 100% was reported only in a few studies. Alani et al. (2007) showed that the sensitivity of telomerase activity in 50 lung cancers was 100% (15).

Dikmen et al. in two different studies showed that sensitivities of telomerase activity in the malignant bronchoalveolar lavages and pleural effusions were 72.7% and 82.5%, respectively (8,9). In another study on 65 pleural effusions, this value was 90% (13). Some other studies reported the sensitivity of telomerase activity to be above 80% (16-19). The modest sensitivity in our literature review was related to Spangler et al's study which was performed on pleural effusions of dogs and cats; They showed that the sensitivity of telomerase activity was similar to cytologic examination (both 50%) with lower specificity (83% vs. 100%) (20).

In the present study we found that there was no false-negative results in telomerase activity of malignant pleural effusions. However, some other studies reported 18-31% false-negative results (9,21, 22). False-negative results can occur due to existence of polymerase inhibitors such as hemoglobin, mucin, presence of proteases and RNase in samples, limited number of cells in samples, or inactivation of telomerase in the freezing or preparation process (23, 24).

False-positive result existed only in one benign pleural effusion (11%) in a patient with TB. This may be due to the presence of lymphocytic inflammatory cells. Other studies reported false-positive results in a range of 10 to 15% (9). However in case of TB, some studies showed that false-positive results may occur in up to 40% ; Although telomerase assay can be helpful in the malignant effusions, presence of lymphocytes and mesothelial cells can cause false-positive results (25).

In our study, specificity of telomerase activity for lung cancer was 88.9%. Indeed, this shows that it will detect 90% of cancer-free patients with benign pleural effusions. Dikmen et al. in two separate studies showed that specificities of telomerase activity were 85.7% and 80.4% (8,9). One study reported higher (94%) specificity for telomerase activity in pleural effusions (13).

Positive and negative predictive values are other parameters of diagnostic value which show probability of disease or healthy status of a patient with positive or negative test results, respectively. Both values were considerably high in our study (95% and 100%, respectively). In Dikmen et al. study, positive predictive value of telomerase activity in 29 bronchoalveolar lavages was 94%, but negative predictive value (50%) was significantly lower than our study (9).

Diagnostic accuracy of telomerase activity for lung cancer was 96.4%. This finding supports usefulness of telomerase activity in diagnosis of lung cancer. It is important to note that to our knowledge, all similar studies reported lower diagnostic accuracy for telomerase activity in malignancies (8-10, 13, 16, 23-30).

In this study, number of patients was relatively low due to limited numbers of available patients with lung cancers in our setting. Further studies with a larger sample size and similar sex distribution in both groups of malignant and benign effusions are recommended.

In conclusion, telomerase is a valuable biomarker for diagnosis of pulmonary malignancies, and measurement of telomerase activity is a useful diagnostic tool for lung and pleural cancers with high diagnostic values. This test needs only a few cells for detection of malignancy and is performed in a simple

manner. Therefore, we suggest that this test should be used in conjunction with other methods, such as cytology for diagnosis of lung cancers.

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