Comparison of Telomerase Activity between Malignant and Tuberculous Pleural Effusions

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Objective: *To determine if telomerase activity can differentiate malignant from tuberculous pleural effusions.*
Design: *Telomerase activity in malignant and tuberculous pleural effusions was measured in a blinded manner using a PCR-based telomeric repeat amplification protocol (TRAP) assay.*
Material and Method: *Fifty-two patients with lymphocytic exudative pleural effusions were identified on thoracocentasis over a period of 18 months*
Results: *Telomerase activity was detected in 34% of malignant pleural fluid samples and 50% of tuberculous pleural effusions. The positive rate of telomerase activity was 30.7% for primary lung cancer and 37.5% for metastatic pleural effusion. The sensitivity and specificity of telomerase activity assay were extremely low (35.7% and 52.9%, respectively), compared with that of cytological examination (52.6% and 65.4%, respectively). Moreover, the diagnostic accuracy of telomerase activity in combination with cytology was even lower than cytological examination alone (46.7% vs. 60%, respectively). This finding was in contrast to previous reports and demonstrated that the detection rate of telomerase activity in tuberculous pleural effusions was greater than that observed in malignant pleural exudates.*
Conclusion: *Telomerase activity does not appear to be a useful marker for differentiating malignant from tuberculous effusions*

Keywords: *Telomerase activity, Malignancy, Tuberculosis, Pleural effusion*

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Lymphocytic pleural effusions often create a dilemma in clinical practice, especially in terms of distinguishing between benign and malignant causes. In addition, differentiating between malignant and tuberculous pleural effusion has important prognostic and therapeutic implications. Although cytological diagnoses have high specificity, the sensitivity ranges between 40% and 80%[1]. The low sensitivity may be due to the presence of scant malignant cells in effusions and the difficulty of distinguishing malignant cells from reactive mesothelial cells. To improve the sensitivity of cytological examination, various adjuvant methods have been proposed, including determination of the level of various tumor markers[2,3], immunocytochemistry with various tumor associated antibodies[4], and chromosome analysis[5]. However, these special techniques have a limited impact in routine cytological diagnosis. It is necessary to develop a feasible new test in order to improve limitations of the morphological approach in pleural effusion.

Telomerase is a specialized reverse transcriptase that directs the synthesis of telomeric repeats (TTAGGG in humans) at chromosome ends[6]. Telomerase appears to play a key role in maintaining telomere length and in replicative senescence. Normal human somatic cells express low or undetectable telomerase activity and are mortal. In contrast, moderate or high telomerase activity is present in the majority of immortal and cancer cells[7,8]. Ectopic expression of telomerase

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activity has been shown to extend the life-span of several normal human cells such as lymphocytes[9-12]. Together these observations support an important role of telomerase in cellular immortalization.

The telomeric repeat amplification protocol (TRAP) assay, a polymerase chain reaction (PCR)-based telomerase assay, is a highly sensitive method for detecting telomerase activity among as few as 1-10 immortal cancer cells and 0.01% positive cells in a mixed population[7]. Some studies have also shown the telomerase activity measured by the TRAP assay might be a potentially useful and noninvasive method to detect malignancy in body fluid[13-16], especially when used in conjunction with conventional cytological examination[17,18]. However, contradictory reports suggested that the measurement of telomerase activity added little to the diagnostic value for malignant pleural effusions[19,20].

Both $CD4^+$ and $CD8^+$ T cells are activated[21-23] and retain proliferative capacity[24] upon mycobacterial tuberculosis infection. The proliferative capacity of T cells is due to the upregulation of telomerase activity[25]. Telomerase activity in peripheral blood T cells is detectable at low levels or undetectable at resting conditions, but upregulated several hundred-fold during activation[26-28]. This observation is also consistently demonstrated by a recent study showing that proliferative lymphocytes in pleural effusions express moderate levels of telomerase activity[29] and suggesting that telomerase reactivity found in lymphocytes may reduce the specificity for malignancy. Although diagnostic implications of telomerase activity have been investigated in malignant pleural effusions, we were aware of only a few reports clarifying the potential use of telomerase activity in differentiating between malignant and tuberculous pleural effusions. In the present study, we investigated whether the detection rate of telomerase activity was higher in tuberculous pleural effusions than malignant pleural exudates. In conclusion, determination of telomerase activity in pleural effusion did not provide diagnostic value in differentiating between malignant and tuberculous pleural effusions because of the unacceptably high number of false-positive cases seen in tuberculosis.

Material and Method

Patients and pleural fluid samples

Fifty-two patients presenting with lymphocytic exudative pleural effusions were included in this study. Exclusion criteria were a positive serologic test for HIV and the use of immunosuppressive agents. Written informed consent was obtained from each patient, and the study was approved by the Ethics Committee of the Siriraj Hospital. Clinical signs and symptoms and demographic data were recorded. The following three groups of patients were evaluated:

1. Confirmed pleural TB based on the conventional "gold standard," a smear or culture positive for *M. tuberculosis* from pleural fluid and/or histology showing a caseating granuloma.

2. Probable pleural TB based on signs and symptoms (cough, chest pain, and pleural exudation) or histology showing a chronic inflammation without caseating granuloma, with response to treatment or a culture positive for *M. tuberculosis* from sputum or bronchoalveolar lavage associated with a pleural effusion.

3. Malignant pleural effusion was established by cytologic and/or histologic testing.

Study design

We subjected 52 lymphocytic exudative pleural effusion samples to cytological examination. The samples were obtained from 32 men and 20 women. Telomerase activity assay and routine cytological examination were performed independently in a double-blinded manner. When discrepan-cies existed between the cytological diagnosis and the TRAP assay, the specimens were reevaluated.

Pleural biopsy was performed in all individuals associated with a malignant tumor or suspected TB. Patients with undiagnosed pleural effusion despite multiple thoracocentasis, either would undergo pleuroscopic examination or would be treated as TB pleuritis if their clinical features suggested this, and then clinical and radiological responses would be assessed.

Pleural fluid specimens

Cells from 60 ml of fresh pleural effusions were collected by centrifugation at 500 r.p.m. for 10 minutes. The supernatant was removed completely and the residual cells were washed twice with cold phosphate-buffered saline (PBS) at 4°C. The resultant cell pellet was snapfrozen in liquid nitrogen and stored at -80°C until analysis.

Protein extraction

The cell pellets were resuspended in 200 μL ice-cold CHAPS (3- [(3-chlomidopropyl) dimethyl-ammonio]-1-propanesulfonate) lysis buffer including 0.5% CHAPS, 1 mM MgCl2, 5 mM β-mercaptoethanol, 1 mM EGTA, 0.1 mM AEBSF, 10 mM Tris-HCl (pH 7.5)

and 10% glycerol. The mixtures were pipetted thoroughly until the pellet was no longer visible and incubated on ice for 30 minutes. Cell debris was removed by centrifugation for 30 minutes at 12,000 rpm at 4°C. The supernatant was transferred to a fresh tube and the protein concentrations were determined as indicated below. Thereafter the supernatant samples were diluted to a protein concentration of 3 μg/μL with lysis buffer and snap-frozen in liquid nitrogen and immediately stored at -80°C.

Determination of protein concentrations

Protein concentrations were determined using Bradford's reagent (Bio-rad Laboratories, CA, USA). First, a set of five different concentrations (1-5 μg/μL) was made up by dilution of a stock 10 mg/ml BSA solution used as standard. Protein samples were also diluted 1:2 and 1:4 to ensure that the concentration measured was within the range of the assay. To separate wells of a 96-well plate, individual unknown samples and serial concentrations of the standard including a no protein diluent were applied in duplicate. To each well 200 μL of Biorad buffer was added. The absorbance at 600 nm of each sample was measured within 5 minutes using a plate reader. A standard curve of absorbance against concentration was plotted and used to establish the concentration of the unknown samples.

Telomerase assay

The telomeric repeat amplification protocol (TRAP) assay was performed using the TRAP-ezeTM telomerase detection kit (Chemicon International Inc, Cat. No. S7700-kit, CA, USA) according to the manufacturer's instructions. Briefly, two μL of supernatant was added into a 43 μL reaction mix containing 50 μM dNTP, 0.1 mg TS primer (5′-AATCCGTCGAGCA GAGTT-3′) and 1× TRAP buffer (20 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA). After incubation for 30 min at room temperature (25°C), 5 μL of amplifying mix containing 0.1 μg CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) and 2 U Tag-DNA polymerase (Epicenter Madison, WI, USA) were added into PCR tubes. Telomerase products were amplified in 40 PCR cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s in a thermal cycler (Minicycler, MJ Research, Watertown, USA) with the hot start at 50°C for 30 min. Thereafter, 40 μL of the PCR products were loaded onto a 3% agarose gel and resolved by electrophoresis at 70 V for 120 min in 1 × Tris boric acid EDTA (TBE) buffer. The gels were stained with ethidium bromide for 3 minutes and visualized with Image Master ID version 2.0 (Pharmacia Biotech, Sunnyvale, CA).

Adenosine deaminase assay

ADA activity was determined in 1 mL pleural fluid using the colorimetric method described by Giusti and Galanti[30]. A positive result was defined as a value > 45.5 U/L based on the previous analysis of pleural fluid samples with proven TB and non-TB. A positive control sample and two negative control samples for which the ADA value was known were included in each group of samples analyzed.

Data analyses

The sample showing a ladder of products with a six base-pair increment was considered positive. Extracts that revealed only a 36-bp band but no ladder of PCR products were considered negative for telomerase activity. An extract of 10 μL from each sample was incubated at 85°C for 10 min prior to TRAP assay to inactivate telomerase and samples sensitive to heat treatment were considered to contain telomerase activity. Cell extracts provided in the kit were used as positive controls, whereas CHAPS lysis buffer was used as a negative control.

Results

Patient characteristics

The mean age of all patients with malignant pleural effusions was 60.4 ± 2.4 years (range, 38-75 years). Seven pleural effusions were excluded because of indefinite diagnosis. Of the 29 malignant pleural effusions, 13 were adenocarcinoma of the lung, eight were metastatic adenocarcinoma; breast cancer (3 cases), ovarian mucinous cystadenocarcinoma (1 case), cervical cancer (1 case), gastric cancer (1 case), nasopharynx (1 case), malignant lymphoma (1 case), and eight were unknown primary sites (Table 1). Eight, nine and ten malignant pleural effusions were confirmed by pleural cytology, histopathology, and both, respectively. Four cases required multiple thoracocentasis for definite diagnosis of malignant pleural effusion. Cytologic examination confirmed the malignant nature of 20 (68.2%) samples in the malignant group.

Of the 16 patients with tuberculous pleural effusions (mean age, 50.4 ± 4.9 years) (Table 2), 11 were definitely diagnosed by either histopathology or demonstration of mycobacterium in their pleural fluid (i.e. positive AFB or culture for mycobacterium tuberculosis), while five patients were diagnosed as

Table 1. Clinicopathological data about telomerase activity in malignant pleural effusions

Patient No./age, Yr/Sex	Underlying malignancy	Cytology	Histology activity	Telomerase
1/76/M	Unknown	+	-	-
2/38/F	Unknown	+	-	-
3/54/F	Ovary	+	-	+
4/69/F	Unknown	+	-	+
5/66/F	Lung	+	-	-
6/61/F	Breast	+	-	-
7/75/F	Unknown	+	+	+
8/51/F	Cervix	+	+	-
9/58/M	Unknown	+	+	-
10/69/M	Lung	+	+	-
11/44/M	Lung	+	+	+
12/73/M	Lung	+	+	+
13/75/F	Colon	+	+	+
14/54/M	Lung	+	+	+
15/52/F	Breast	-	+	+
16/63/F	Unknown	-	+	-
17/42/F	Breast	-	+	-
18/70/F	Unknown	-	+	-
19/77/M	Lung	-	+	-
20/70/M	Unknown	-	+	+
21/57/M	Lung	-	+	-
22/52/M	Lung	+	+	-
23/68/M	Lung	+	-	-
24/60/M	Lung	-	+	-
25/59/F	Lung	-	+	-
26/68/F	Lung	+	-	+
27/74/F	Lung	+	+	-
28/27/M	Nasopharyx	+	-	-
29/51/M	Lymphoma	+	-	-

probable tuberculous pleuritis (positive culture for TB in sputum (1 case), and bronchial lavage fluid (1 case), and the remaining three had clinical and radiological responses to anti-tuberculous drugs).

Lymphocyte counts were significantly greater in tuberculous effusions than malignant pleural exudates (1991.2 ± 222.9 vs 992.8 ± 144.7, $p = 0.0012$). In contrast, malignant effusion samples contained a significantly higher amount of mesothelial cells than that seen in tuberculous effusions (1.46 ± 0.43 vs 12 ± 3.3, $p = 0.004$).

Telomerase activity in pleural effusions

Of the 29 malignant specimens, only 10 (34%) of the pleural effusions had measurable levels of telomerase activity (Table 1). Only eight of 20 cytology-positive pleural effusions (40%) exhibited telomerase activity, whereas cytology-negative and telomerase-positive malignant effusions were 28% (Fig. 1). About 50% of cytology-positive malignant pleural effusions in conjunction with telomerase activity were demonstrated in patients with primary lung cancer, and 50% were observed in metastatic malignant pleural effusions including unknown primary malignancy. Nine out of 13 (69.3%) lung cancer-associated malignant pleural effusions were negative for telomerase activity (Fig. 2). Among malignant pleural effusions related to primary lung cancer, telomerase activity in combination with positive cytology was only 45%, whereas positive cytology in the absence of telomerase activity was higher (55%) (Fig. 2). Comparing the tuberculous pleuritis group, the sensitivity, specificity, PPV, NPV, and accuracy of the TRAP assay was 35.7%, 52.9%, 55.6%, 33.3%, and 42.2%, respectively.

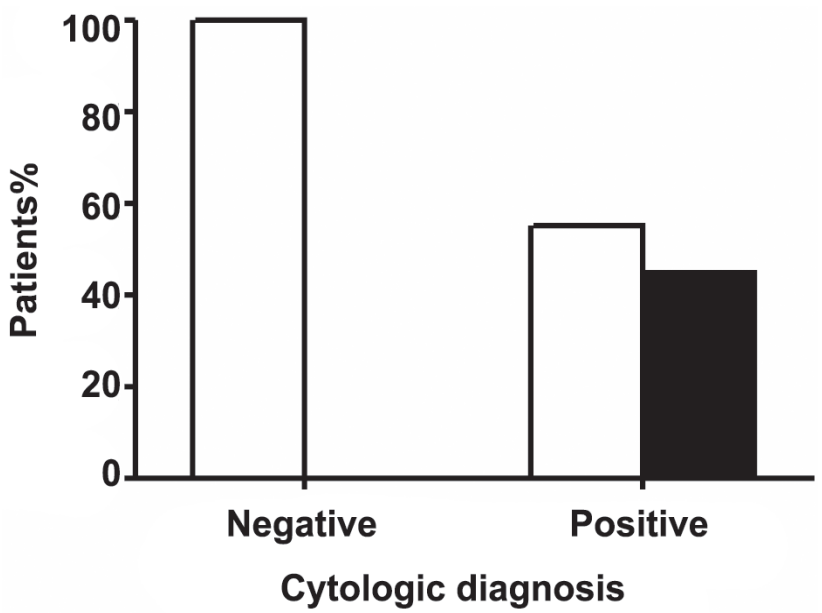

Fig 1. Positive (■) and negative (□) telomerase activity in pleural fluid specimens diagnosed as malignant in routine cytology

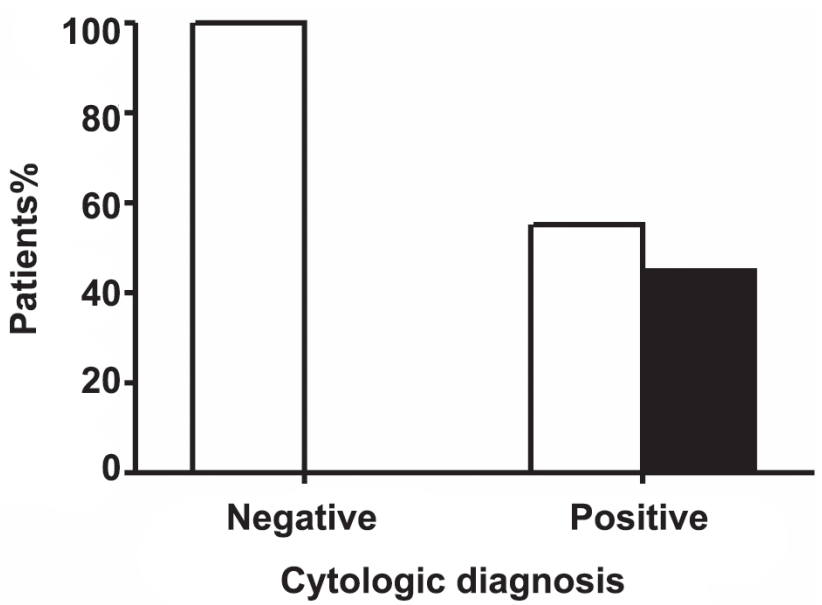

Fig 2. Positive (■) and negative (□) telomerase activity in pleural fluid specimens diagnosed as primary lung cancer in routine cytology

In contrast, telomerase activity was positive in eight of 16 (50%) tuberculous pleural fluid specimens (Table 2). Only three out of 8 (27.5%) tuberculous pleural exudates with positive telomerase activity exhibited ADA levels higher than the laboratory cutoff point (mean ± s.e.m, 45.3 ± 1.28), which was almost equivalent to the number of patients with telomerase negative tuberculous pleural effusion (mean ± s.e.m,

Table 2. Clinicopathological data about telomerase activity in tuberculous pleural effusions

Patient No./age, Yr/Sex	Mycobacterial diagnosis	Histologic diagnosis	Telomerase activity
1/70/M	+	+	-
2/53/M	+	-	-
3/38/M	+	-	-
4/38/F	-	+	+
5/56/M	-	+	-
6/47/F	-	+	+
7/81/M	-	+	-
8/23/F	-	-	-
9/35/M	-	+	+
10/55/M	+	+	+
11/23/M	-	-	+
12/36/M	-	+	+
13/79/M	-	+	+
14/74/M	-	+	-
15/71/M	-	+	-
16/27/M	-	+	+

Table 3. Diagnostic values of telomerase activity and cytological examination for malignant pleural effusions

	Sensitivity	Specificity	Diagnostic accuracy
Cytological examination	52.6	65.4	60
Telomerase activity	35.7	52.9	42.2
Cytological examination and telomerase activity	63.2	34.6	46.7

Data are presented as %

47.1 $\pm$ 1.41). It seemed that there was no correlation between ADA levels and telomerase activity.

Discussion

The present study proposes that analysis of telomerase activity using TRAP assay may not be useful as an adjunct to cytologic findings in determining malignant pleural effusions and differentiating them from tuberculous pleural effusions. The sensitivity and specificity rates for cytological examination in combination with telomerase activity were 63.2% and 34.6%, respectively, while 35.7% and 52.9% were for telomerase activity alone. Diagnostic accuracies of cytological examination and telomerase activity were 60% and 42.2%, respectively (Table 3). Interestingly, the rate of telomerase positivity detected in patients with tuberculous pleuritis appeared to be higher than that demonstrated in malignant pleural effusions.

Our data revealed that the diagnostic yield of cytological examination showed a tendency toward significance between the malignant and TB groups. When we compared these diagnostic tools, we observed no significant correlation between the number of false negative samples of cytological examination in those diagnosed with telomerase activity which was malignant (2 cases) and the opposite, false-negative cases of telomerase activity in those diagnosed with cytological examination (10 cases). Likewise, previous reports had similar data showing that no correlation existed between cytological examination and telomerase activity[31,32]. Thus, we may reasonably suggest that the detection of telomerase activity in pleural effusions, in contrast to bronchial lavage samples[33], may not provide a greater rate of definitive diagnosis for malignancy compared with cytological examination. The sensitivity of cytological examination in combination

with telomerase activity was not significantly greater than that of cytology alone in our study as shown in Table 3 (63.2% vs 52.6%, respectively).

The rate of false negative samples for telomerase activity in previous reports ranged between 18 and 31%[31,32,34,35] and 63.3% in our observation. This may be attributed to several factors. Taq polymerase inhibitors such as hemoglobin, mucin, RNAse and protease may play a part in the medium with a resultant negative telomerase activity[36]. In addition, the lack of an adequate number of cells extracted from the sample, and inactivation of telomerase enzymes during the freezing and thawing procedures may result in a negative telomerase activity. Apart from the above-mentioned interfering factors, malignant cell types may account for the telomerase-negative phenotype. Adenocarcino-matous cancers have lower telomerase activity than other cell types in particular in squamous cell carcinoma[32,33]. This speculation might be supported by our observation that only 4 (44.4%) pleural fluid samples from patients with adenocarcinoma of the lung cancer were telomerase-positive, possibly explaining the high negative rate of telomerase activity observed in the present study, and suggesting that cancers with negative telomerase maintain their proliferative capacity by alternative lengthening of the telomeres mechanism, a recombination-mediated DNA replication process[37,38].

Very few studies have clarified the potential use of telomerase activity in distinguishing malignant from tuberculous pleural effusions with lymphocytic predominance, which is often a dilemma in clinical practice. The present study demonstrated that the rate of telomerase activity was greater in tuberculous pleuritis compared with malignant disease (50% vs 34.4%, respectively). It suggested that analysis of telomerase activity in pleural effusions did not show any considerable potential to differentiate between malignant and tuberculosis cases. It has been recently suggested that lymphocytes are activated and induced to be proliferative upon tuberculous infection[29,39]. Moreover, the activated T cells show a several-hundred fold increase of telomerase activity as compared with the resting state[27,28]. These results allow us to speculate that the contamination of telomerase activity from active lymphocytes in tuberculous pleural effusions might weaken the diagnostic value of telomerase activity assay for malignant pleural effusions, resulting in low specificity of telomerase activity as evidenced by our study. This hypothesis is supported by the current findings that lymphocyte-rich effusions were significantly greater in tuberculosis cases compared with malignant pleural effusions. Whether the proliferation capacity of these lymphocytes in tuberculous pleural effusions is associated with high telomerase activity remains an interesting and relevant issue that requires further study, although this association is true of the previous *in vitro* study[40]. Nonetheless, on the basis of our data, it suggests that telomerase activity has its limitation in cases of differentiation between tuberculous and malignant pleural exudates.

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การเปรียบเทียบเทโลเมอเรสแอคทิวิตี้ของสารน้ำในช่องเยื่อหุ้มปอดจากมะเร็งและวัณโรค

กิตติพงศ์ มณีโชติสุวรรณ, อรุณ เลิศวรวิวัฒน์, แจ่มศักดิ์ ไชยคุนา, วัลลา วามนัฐจินดา

Telomerase เป็น reverse transcriptase ที่สำคัญเกี่ยวข้องกับการรักษาความยาวของ telomere ของเซลล์ที่กำลังแบ่งตัว มักตรวจพบ Telomerase activity ในเซลล์มะเร็งเกือบทุกชนิด ผู้ทำการวิจัยมีวัตถุประสงค์ที่จะศึกษาถึง Telomerase activity ในน้ำช่องเยื่อหุ้มปอดที่มีสาเหตุจากโรคมะเร็งและวัณโรค ผู้ศึกษาใช้วิธี PCR-based telomeric repeat amplification protocol (TRAP) assay เพื่อวัดระดับของ Telomerase activity ในน้ำช่องเยื่อหุ้มปอดที่มีลักษณะเป็น exudate และส่วนใหญ่ของเม็ดเลือดขาวเป็น lymphocytes (lymphocytic exudative pleural effusion) จากผู้ป่วยทั้งหมดจำนวน 52 ราย โดยที่ผู้ทำการตรวจหา Telomerase activity ไม่ทราบการวินิจฉัยโรคของผู้ป่วยที่อยู่ในการศึกษา ผลการศึกษาพบว่าร้อยละ 38 ของผู้ป่วยมะเร็ง และร้อยละ 50 ของผู้ป่วยวัณโรคให้ผลบวกของ Telomerase activity ในน้ำช่องเยื่อหุ้มปอด อัตราการตรวจพบ Telomerase activity ในกลุ่มผู้ป่วยมะเร็งปอดคิดเป็นร้อยละ 30.7 ในกลุ่มผู้ป่วยมะเร็งชนิดแพร่กระจายจากอวัยวะอื่น ๆ คิดเป็นร้อยละ 37.5 ค่าความไวและความจำเพาะ (sensitivity และ specificity) ของการตรวจ Telomerase activity ในกลุ่มผู้ป่วยมะเร็งคิดเป็นร้อยละ 35.7 และ 52.9 ตามลำดับ ซึ่งต่ำกว่าเมื่อเปรียบเทียบกับค่าความไวและความจำเพาะของการตรวจทางเซลล์วิทยาซึ่งคิดเป็นร้อยละ 52.6 และ 65.4 ตามลำดับ ความแม่นยำของการตรวจ Telomerase activity ร่วมกับการตรวจทางเซลล์วิทยาสำหรับการวินิจฉัย โรคมะเร็งในผู้ป่วยที่มีน้ำในช่องเยื่อหุ้มปอดต่ำกว่าการตรวจทางเซลล์วิทยาอย่างเดียว (คิดเป็นร้อยละ 46.7 และ 60 ตามลำดับ) จากข้อมูลข้างต้นทำให้สรุปได้ว่าการตรวจ Telomerase activity ในน้ำช่องเยื่อหุ้มปอดไม่มีประโยชน์ ทางคลินิคในการนำมาใช้แยกโรคมะเร็งจากวัณโรค