Eur Respir J 2003; 22: 422–426
DOI: 10.1183/09031936.03.00001303
Printed in UK – all rights reserved

Copyright ©ERS Journals Ltd 2003
European Respiratory Journal
ISSN 0903-1936

Diagnostic implications of telomerase activity in pleural effusions

G. Dikmen*, E. Dikmen#, M. Kara#, E. Şahin¶, P. Doğan*, N. Özdemir¶

Diagnostic implications of telomerase activity in pleural effusions. G. Dikmen, E. Dikmen, M. Kara, E. Şahin, P. Doğan, N. Özdemir. ©ERS Journals Ltd 2003.
ABSTRACT: The aim of the present study was to investigate the diagnostic efficacy of telomerase activity for discrimination of malignant and benign pleural effusions.

Pleural effusions were collected from 109 consecutive patients in whom the diagnosis was confirmed with cytological and/or histological examinations. Cytological samples were classified as malignant (n=63) and benign (n=46). Telomerase activity was determined with the polymerase chain reaction-based telomeric repeat amplification protocol assay.

Telomerase activity was detected in 52 (82.5%) and nine (19.6%) samples from the malignant and benign groups, respectively, which was a significant difference. The sensitivity rate of cytological examination when combined with telomerase activity (92.1%) was significantly greater than that of cytological examination alone (53.9%). The sensitivity and specificity of telomerase activity were 82.5 and 80.4%, respectively. Diagnostic accuracy of telomerase activity was 81.6%.

Telomerase activity is a highly sensitive diagnostic biomarker for malignancy and may be used as an adjunct to cytological findings in determining malignant pleural effusions.
Eur Respir J 2003; 22: 422–426.

*Dept of Biochemistry, Faculty of Medicine, Hacettepe University, Ankara, #Dept of Thoracic Surgery, Faculty of Medicine, Kirikkale University, Kirikkale, and ¶Dept of Thoracic Surgery, Faculty of Medicine, Ankara University, Ibn-i Sina Hospital, Ankara, Turkey.

Correspondence: E. Dikmen, Bulbulderesi Caddesi 65/8, TR-06660, Kucukesat, Ankara, Turkey.
Fax: 90 3182252819
E-mail: erkandikmen69@hotmail.com

Keywords: Pleural effusion, malignancy, telomerase

Received: January 7 2003
Accepted after revision: April 24 2003

This study was supported by the Research Fund of the Faculty of Medicine, University of Hacettepe, Ankara, Turkey (project no. 1-01-02.101.021).

Pleural effusions with unknown aetiology are frequent and often create a dilemma in clinical practice. Although the diagnostic yield can reach up to 87% with more invasive thoracoscopic procedures, diagnosis of pleural effusions can be established in 33–72% of patients by conventional diagnostic methods [1–3]. Numerous procedures, including immunocytochemistry, chromosome analysis, tissue culture techniques, deoxyribonucleic acid (DNA) flow or image cytometry, and cell imaging combined with immunocytochemistry have been proposed for the differential diagnosis of pleural effusions to improve the sensitivity of cytological examination [4]. Despite these sophisticated diagnostic methods, definitive diagnosis of pleural effusions may still be difficult. In addition, the nature of pleural effusions is of particular importance in lung cancer patients and needs to be clarified, as a co-existing malignant pleural effusion implies an advanced stage, which precludes a possible surgical resection [5].

Telomeres are specialised structures at the ends of the chromosomes in eukaryotic cells that provide genomic stability and permit complete replication. They shorten with each cell division, resulting in cellular senescence, and they limit the reproductive capacity of the cell, which is thought to be the "biological clock" of the cell. Telomerase is a ribonucleoprotein that compensates the telomeric loss by synthesising telomeric DNA and is believed to play an important role in the development of malignancy [6]. Telomerase expression has been shown in various human tumours and immortalised cell lines [7, 8]. Furthermore, the assay of telomerase activity has been suggested to be a potential biomarker for cancer detection in body cavity fluids [4, 9]. This study was conducted to determine the use of telomerase expression in diagnosing malignant pleural effusions and the results were compared with those of the cytological examination.

Methods

Patients and pleural effusion samples

Pleural fluid samples were collected from 109 consecutive patients who underwent diagnostic or therapeutic thoracentesis in the Ibn-i Sina Hospital of Ankara University, Ankara, and in the Hospital of Kirikkale University, Kirikkale, Turkey, between January 2001 and March 2002. The patients included 69 males and 40 females with a mean age of 54.0±12.6 yrs (range 17–77 yrs). Diagnosis was confirmed with cytological and/or histological examinations. Cytological samples were classified as malignant or benign. The median follow-up of all patients was 13 months (range 7–22 months).

The malignant group consisted of 63 patients with malignant pleural effusion. The patients included 36 males and 27 females with a mean age of 56.1±12.0 yrs (range 17–72 yrs). Definitive diagnosis was confirmed with invasive procedures such as closed or thoracoscopic pleural biopsy in patients lacking definitive cytological diagnosis. The most common histological diagnosis was malignant mesothelioma in 20 (31.7%) patients, followed by 18 (28.5%) patients with lung cancer, 10 (15.8%) patients with metastatic breast cancer, 10 (15.8%) patients with metastatic ovarian cancer, three (4.7%) patients with metastatic pancreas cancer and two (3.1%) patients with malignant lymphoma. Of the 18 lung cancer patients, 16 had nonsmall-cell lung cancer (NSCLC) and two had small cell lung cancer (SCLC) (table 1).

The benign group consisted of 46 patients with benign pleural effusion. The patients included 33 males and 13 females with a mean age of 51.0±12.9 yrs (range 26–77 yrs). Diagnosis was made on the basis of clinical and radiological features, response to therapy and cytological examinations of pleural fluids. In addition, diagnosis was confirmed in 24 patients with closed or thoracoscopic pleural biopsy and thoracotomy. None of the patients had co-existing or previous cancer disease and none developed malignancy during the follow-up period. Definitive diagnosis was parapneumonic empyema in 19 (41.3%) patients, congestive heart failure in 11 (23.9%) patients, post-traumatic effusion in five (10.8%) patients, tuberculosis in five (10.8%) patients, acute pancreatitis in four (8.6%) patients and nephrotic syndrome in two (4.3%) patients.

Determination of telomerase activity

Telomerase activity was determined with a polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) assay. In this primer extension assay telomerase synthesises telomeric repeats onto oligonucleotide primers and the extension products then serve as the templates for PCR amplification [10].

The pleural effusion samples were transported to the laboratory within 3 h of collection. After centrifugation at 1,400×*g* for 10 min, the suspended cells were separated and washed twice with ice-cold 10 mM phosphate-buffered saline (pH 7.4). The cell pellets (100 μL) were treated with 200 μL ice-cold CHAPS (3-{[3-chlomidopropyl]-dimethyl-ammonio}-1-propanesulfonate) lysis buffer including 0.5% CHAPS, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM EGTA (ethylene glycol bis [β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid), 0.1 mM AEBSF (4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochlorine), 10 mM Tris-HCl (pH 7.5) and 10% glycerol. Samples were kept on ice for 30 min and then centrifuged at 13,000×*g* for 30 min at 4°C. Supernatants were removed carefully and the protein concentrations were determined by the Bradford method [11]. The supernatant samples were diluted to a concentration of 3 μg·μL protein^{-1} with lysis buffer and stored at -70°C.

PCR-based TRAP assay, as described previously by KIM *et al.* [10] was used to measure telomerase activity. Two μL of supernatant (3 μg protein·μL^{-1}) was added into a 43 μL reaction mix containing 50 μM dNTP, 0.1 μg TS primer (5′-AATCCGTCGAGCAGAGTT-3′) and 1×TRAP Buffer (20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA). After incubation for 30 min at room temperature (25°C), 5 μL amplifying mix containing 0.1 μg CX primer (5′-CCCTTACCCTTACCCTTACCC-TAA-3′) and 2 U Tag-DNA Polymerase (Epicentre Madison, WI, USA) were added into PCR tubes. Telomerase products were amplified in 33 PCR cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 90 s in a thermal cycler (Perkin Elmer 2400; Perkin Elmer, Norwalk, CT, USA) with the hot start at 90°C for 90 s.

Thereafter, 20 μL of the PCR products were loaded onto a 12% polyacrylamide nondenaturing gel and resolved by electrophoresis at 200 V for 120 min in 0.5×Tris-borate-ethylenediamine tetraacetic acid buffer. The gels were stained with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) for 30 min and visualised.

Evaluation of telomerase activity

The samples showing a ladder of products with a six base-pair increment were considered to be positive. An extract of 2 μL from each sample was treated with 1 μg deoxyribonuclease-free ribonuclease (RNase) at 37°C for 60 min prior to the TRAP assay and samples sensitive to RNase treatment were considered to contain telomerase activity. HeLa cells were used as positive controls, whereas CHAPS lysis buffer were used as negative controls (fig. 1). Telomerase activity assays and cytological/histological examinations were performed independently in a blinded manner.

Statistical analysis

Results were analysed with the Chi-squared test. The sensitivities of cytological examination and telomerase activity were compared with the McNemar test in the malignant group. Data were expressed as mean±SD. A p-value of <0.05 was considered statistically significant.

Results

Cytological examination confirmed the benign nature of all 46 (100%) samples in the benign group, however, cytological examination confirmed the malignant nature of 34 (53.9%) samples in the malignant group (p<0.0001). Telomerase

Table 1. – Characteristics of pleural effusions with respect to cytological features and telomerase activities

	Subjects	Positive diagnostic cytology	Positive telomerase activity
Malignant pleural effusion	63	34 (53.9)	52 (82.5)
Malignant mesothelioma	20	8 (40)	18 (90)
NSCLC	16	10 (62.5)	12 (75)
SCLC	2	2 (100)	2 (100)
Breast cancer	10	6 (60)	8 (80)
Ovarian cancer	10	6 (60)	9 (90)
Pancreas cancer	3	1 (33.3)	2 (66.6)
Malignant lymphoma	2	1 (50)	1 (50)
Benign pleural effusion	46	46 (100)	9 (19.6)
Parapneumonic empyema	19		6 (31.5)
Congestive heart failure	11		
Posttraumatic effusion	5		
Tuberculosis	5		3 (60)
Acute pancreatitis	4		
Nephrotic syndrome	2		

Data are presented as n (%). NSCLC: nonsmall-cell lung cancer; SCLC: small-cell lung cancer.

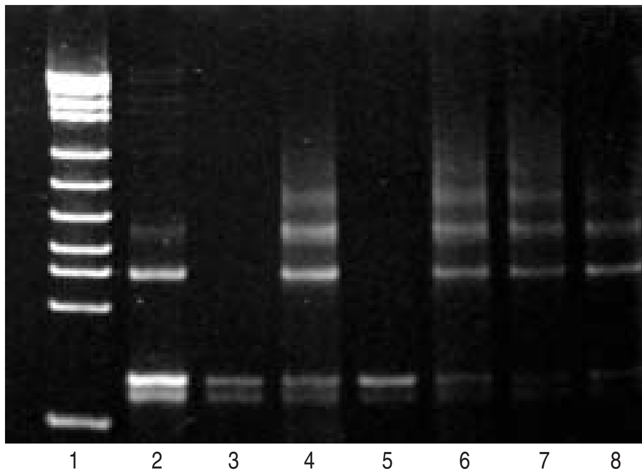

Fig. 1. – Detection of telomerase activity in the pleural effusion samples by telomeric repeat amplification protocol (TRAP) assay. Lane 1: marker (pUC 18 Msp I,26-SOlbp); Lane 2: positive control, HeLa cells; Lane 3: negative control (CHAPS lysis buffer); Lane 4: telomerase (+) metastatic ovarian cancer; Lane 5: telomerase (-) empyema; Lane 6: telomerase (+) lung cancer; Lane 7: telomerase (+) malignant mesothelioma; Lane 8: telomerase (+) lung cancer.

activity was detected in 52 (82.5%) and nine (19.6%) samples from the malignant group and the benign group, respectively, which was a significant difference ($p<0.0001$) (fig. 2). Among the samples with negative telomerase activity in the malignant group, four had NSCLC, two had malignant mesothelioma, two had metastatic breast cancer, one had metastatic ovarian cancer, one had metastatic pancreas cancer and one had malignant lymphoma. Among the samples with positive telomerase activity in the benign group, three had tuberculosis and six had empyema.

Out of 29 pleural effusions without a definitive cytological diagnosis in the malignant group, 24 (82.8%) showed positive telomerase activity, whereas cytological examination was diagnostic in six (54.5%) out of 11 malignant samples with negative telomerase activity in the malignant group ($p=0.002$). The sensitivity and specificity rates for cytological examination were 53.9 and 100%, while these rates were 82.5 and 80.4% for telomerase activity, respectively. Diagnostic yield was achieved in 58 (92.1%) patients in the malignant group

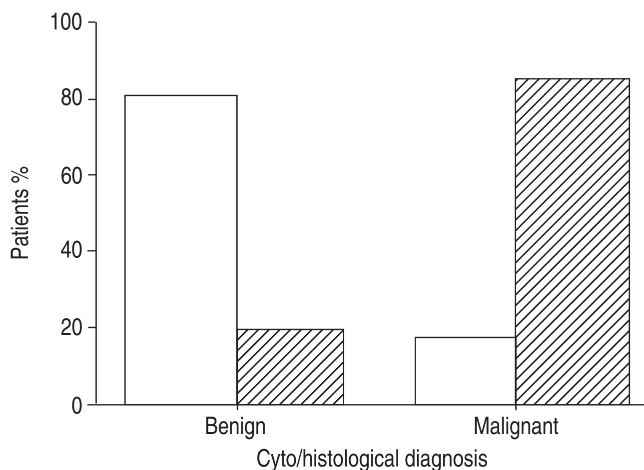

Fig. 2. – Positive (▨) and negative (□) telomerase activity in benign and malignant pleural effusions. $p<0.0001$ when comparing the telomerase ratio between the benign and malignant groups.

Table 2. – Diagnostic values of telomerase activity and cytological examination

	Sensitivity	Specificity	Diagnostic accuracy
Cytological examination	53.9	100	73.4
Telomerase activity	82.5	80.4	81.6
Cytological examination and telomerase activity	92.1	100	95.4

Data are presented as %.

when cytological examination was combined with telomerase activity, whereas 34 (53.9%) of patients in the malignant group could be diagnosed with cytological examination alone, which was a significant difference ($p<0.0001$). Telomerase activity had a positive predictive value of 0.85 and negative predictive value of 0.77. Diagnostic accuracies of cytological examination and telomerase activity were 73.4 and 81.6%, respectively (table 2).

Discussion

Thoracentesis might be the first and most effective diagnostic procedure in the diagnosis of primary or metastatic pleural malignancy with pleural effusion. It may also provide some relief from the pulmonary compromise created by the restrictive effect of the effusion. The diagnostic methods applied to pleural effusions are cell count with differential cytology, direct examination and cultures for bacteria, fungi, acid-fast bacilli, lactate dehydrogenase and protein with fluid to serum ratios and glucose content [2]. However, accurate diagnosis of pre-assumptive malign effusions may sometimes be difficult with these conventional methods.

Cytological diagnosis may be established in 53% of patients with a single thoracentesis, in 64% with two thoracenteses, in 69% with three thoracenteses and it may reach up to 72% with four or more independent samples [3]. Similarly, the current data show that the rate of definitive cytological diagnosis obtained from single samples was 54% in malignant pleural effusions. Conversely, some malignancies, such as Hodgkin's disease, show a lesser rate of diagnostic cytology (23%), while the cytological diagnostic yield ranges 63–73% in lung, metastatic breast and ovarian cancers [12]. Despite the fact that the overall rate for definitive cytological diagnosis was 54% in this study, cytological diagnostic yield was 66% in lung cancer and 60% in either metastatic breast or ovarian cancers, which is consistent with these findings.

Unless the diagnosis is apparent after the first thoracentesis, a second thoracentesis is indicated and should even be accompanied by closed pleural biopsy. However, pleural biopsy has a greater risk of complications than simple thoracentesis and should be reserved for more difficult cases in which the initial thoracentesis and cytological examination fails to yield a diagnosis [2]. The diagnostic yield of pleural biopsy may reach up to 81–90% when combined with cytological examination, however, the diagnostic value of pleural biopsy alone is still not superior to that of cytology [3, 13]. The current data revealed that the diagnostic accuracy of the combined methods was 95% and was significantly greater than that of either cytological examination alone ($p<0.0001$) or the above rate.

When two or more studies of the pleural fluid including closed pleural biopsy fail to provide a diagnosis, a more invasive procedure such as thoracoscopic biopsy is probably the optimal choice because it has a lower risk than thoracotomy and has a diagnostic yield of 87% [1]. However,

thoracoscopic procedures require general anaesthesia and have greater morbidity rates compared with a simple thoracentesis in this group of compromised patients with limited cardio-respiratory functions. Thus, new and less invasive approaches, possibly detection of a "tumour biomarker" should be developed as an alternative method to enhance the sensitivity of cytological examination in the diagnosis of malignant pleural effusions.

Numerous biomarkers have been investigated to differentiate malignant pleural effusions from benign pleural effusions. Among these, carcinoembryonic antigen appears to be the most sensitive biomarker, with a sensitivity rate ranging 25–57% [2, 14]. Thus, more reliable biomarkers, such as the assay of telomerase activity, have been under investigation to detect malignancy in pleural effusions [4, 9]. Telomerase is a reverse transcriptase enzyme that stabilises telomere length by adding hexameric (TTAGGG) repeats to the telomeric ends of the chromosomes by using its own ribonucleic acid (RNA) as a template and compensates the continued erosion of telomeres [15, 16]. Telomerase expression has been detected in various human cancers suggesting that telomerase may be a marker for cellular immortalisation and may indicate the proliferation of tumour cells [7].

Recent data showed positive telomerase activity in >85% of various human malignancies with the use of the TRAP assay, as described previously by Kim *et al.* [10]. Furthermore, telomerase expression has been shown to be a potential diagnostic biomarker in the fluids of body cavities, however, the present authors are aware of only a few reports investigating the telomerase activity with special reference to pleural effusions [6, 17]. These reports showed that the sensitivity of telomerase activity in malignant pleural effusions were 67 and 91%, respectively. The current data revealed a similar rate of 82.5%, which falls between this range and is consistent with these reports. Thus, the authors may reasonably suggest that telomerase expression in pleural effusions is a highly sensitive diagnostic biomarker for malignancy.

The lack of telomerase activity in malignant pleural effusions was 17.5% in this study. False-negative results for telomerase activity may be attributed to several factors, such as Taq polymerase inhibitors (haemoglobine), RNAse and protease used in the sample [7, 18]. In addition, telomerase activity is closely related to the number of malignant cells, and an adequate number of cells, such as 10,000–100,000 should be extracted from 30–50 mL samples [19]. Hence, a low concentration of tumour cells in samples may also result in negative telomerase activity. Furthermore, the enzyme may be inactivated during the freezing and thawing procedures during tissue extraction [20].

Although low telomerase activity has been shown in mononuclear inflammatory cells and proliferative cells of renewal tissues (such as intestinal crypts, basal cells of the skin, hair follicles and endometrial cells), benign somatic cells usually do not show telomerase expression [6]. Likewise, the rate of positive telomerase activity in nonmalignant cytological samples has been reported to be ~10% in previous reports [9, 17, 21]. Among the 46 benign samples, a total of nine (19.6%) samples, composed of six with empyema and three with tuberculosis effusions, showed telomerase expression in the present study. The intense infiltration of lymphocytes and leukocytes in tuberculosis effusions and empyemas, respectively, might explain the false-positive telomerase activity in these samples [22]. However, the specificity of telomerase activity was 80.4% in the current study and this finding supports the proposal that telomerase activity is a reliable diagnostic method in the differential diagnosis of pleural effusions.

As the conventional TRAP assay is insufficient for quantitative analysis and requires post-PCR manipulation, such as polyacrylamide gel electrophoresis or enzyme-linked immunosorbent assay, it is laborious and time consuming. New techniques are needed for accurate quantification of telomerase activity in large-scale samples in a short time. In the real-time quantitative (RTQ)-TRAP method, the PCR reaction and data analysis are done simultaneously. This makes the telomerase activity assay not only simpler and faster but also removes the carry-over contamination [23]. Precise quantification of telomerase activity is required for the development of anti-telomerase therapy in the future.

In conclusion, telomerase expression is a highly sensitive diagnostic method for malignant pleural effusions. Diagnostic yield of malignant pleural effusions will significantly increase, provided that telomerase activity is used in conjunction with cytological examination in pleural malignancies. Further studies should be carried out to determine the accurate quantification of telomerase expression levels to identify the reference values for differentiation of malignant from benign pleural effusions.

References

1. Yim AP, Chung SS, Lee TW, Lam CK, Ho JK. Thoracoscopic management of malignant pleural effusions. *Chest* 1996; 109: 1234–1238.
2. Hausheer FH, Yarbro JW. Diagnosis and treatment of malignant pleural effusion. *Sem Oncol* 1985; 12: 54–75.
3. Salyer WR, Eggleston JC, Erozan YS. Efficacy of pleural needle biopsy and pleural fluid cytopathology in the diagnosis of malignant neoplasm involving the pleura. *Chest* 1975; 67: 536–539.
4. Braunschweig R, Yan P, Guilleret I, *et al.* Detection of malignant effusions: comparison of telomerase assay and cytological examination. *Diagn Cytopathol* 2001; 24: 174–180.
5. Sawabata N, Matsumura A, Motohiro A, *et al.* Malignant minor pleural effusion detected on thoracotomy for patients with non-small cell lung cancer: is tumor resection beneficial for prognosis? *Ann Thorac Surg* 2002; 73: 412–415.
6. Dejmek A, Yahata N, Ohyashiki K, *et al.* *In situ* telomerase activity in pleural effusions: a promising marker for malignancy. *Diagn Cytopathol* 2001; 24: 11–15.
7. Shay JW, Wright WE. Telomerase activity in human cancer. *Curr Opin Oncol* 1996; 8: 66–71.
8. Lee JC, Jong HS, Yoo CG, Han SK, Shim YS, Kim YW. Telomerase activity in lung cancer cell lines and tissues. *Lung Cancer* 1998; 21: 99–103.
9. Mu XC, Brien TP, Ross JS, Lowry CV, McKenna BJ. Telomerase activity in benign and malignant cytologic fluids. *Cancer (Cancer Cytopathol)* 1999; 87: 93–99.
10. Kim NW, Piatyszek MA, Prowse KR, *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; 226: 2011–2015.
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 77: 248–254.
12. Anderson CB, Philpott GW, Ferguson TB. The treatment of malignant pleural effusion. *Cancer* 1974; 33: 916–922.
13. Winkelmann M, Pfitzer P. Blind pleural biopsy in combination with cytology of pleural effusions. *Acta Cytol* 1981; 25: 373–376.
14. Hernandez L, Espasa A, Fernandez C, Candela A, Martin C, Romero S. CEA and CA 549 in serum and pleural fluid of patients with pleural effusion. *Lung Cancer* 2002; 36: 83–89.
15. Shay JW, Gazdar AF. Telomerase in the early detection of cancer. *J Clin Pathol* 1997; 50: 106–109.

16. Moyzis RK, Buckingham JM, Cram LS, *et al.* A highly conserved repetitive DNA sequence (TTAGGG)n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* 1988; 85: 6622–6626.
17. Yang CT, Lee MH, Lan RS, Chen JK. Telomerase activity in pleural effusions: diagnostic significance. *J Clin Oncol* 1998; 16: 567–573.
18. Gollahon L, Holt SE. Alternative methods of extracting telomerase activity from human tumor samples. *Cancer Lett* 2000; 159: 141–149.
19. Ramakumar S, Bhuiyan J, Besse JA, *et al.* Comparison of screening methods in the detection of bladder cancer. *J Urol* 1999; 161: 388–394.
20. Zheng PS, Iwasaka T, Yamasaki F, *et al.* Telomerase activity in gynecologic tumors. *Gynecol Oncol* 1997; 64: 171–175.
21. Cunningham VJ, Markham N, Shroyer AL, Shroyer KR. Detection of telomerase expression in fine needle aspirations and fluids. *Diagn Cytopathol* 1998; 18: 431–436.
22. Hiyama K, Hirai Y, Koizumi S, *et al.* Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol* 1995; 155: 3711–3715.
23. Hou M, Xu D, Bjorkholm M, Gruber A. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. *Clin Chem* 2001; 47: 519–524.