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# Telomerase activity in sputum and telomerase and its components in biopsies of advanced lung cancer

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ABSTRACT

*Purpose:* In this study, we explored the diagnostic utility of sputum telomerase activity as a non-invasive biomarker of lung cancer. In biopsies of lung cancer, the relationship of telomerase activity to telomerase reverse transcriptase (*hTERT*) and telomerase RNA component (*hTERC*) and to *c-Myc* expression was also evaluated.

*Methods:* Paired biopsy and sputum samples were evaluated for telomerase activity by the telomerase repeat amplification protocol (TRAP) assay in 34 cases of lung cancer and in 30 control subjects without any evidence of lung cancer. *hTERT* and *hTERC* transcript expression was evaluated in 42 cases of lung cancer and compared to telomerase activity and *c-Myc* transcript expression.

*Results:* Telomerase activity was present in 85.2% of biopsies and in 67.6% of paired sputum with a good concordance. Three out of the 30 negative controls showed a weak telomerase activity, all of whom had sarcoidosis. Thus, sputum telomerase activity had sensitivity, specificity, Negative Predictive Value and Positive Predictive Value of 67.6%, 90%, 71% and 88.46%, respectively. The *hTERT* levels correlated to the telomerase activity but not to the *c-Myc* oncogene expression.

*Conclusions:* In lung cancer, sputum telomerase activity is a candidate non-invasive biomarker of malignancy.



## 1. Introduction

Lung cancer is the leading cause of cancer deaths and is the most common malignancy worldwide.[1] Despite considerable advances in understanding of molecular biology and pathogenesis of lung cancer, only one out of eight patients diagnosed can be cured at present, the rest die because of widespread metastasis. Recent studies have therefore focused their attention towards the development of reliable early diagnostic markers as well as new therapeutic targets, which could help improve the dismal survival rates of most patients with lung cancer.[2] Of all the markers identified, none have achieved sufficient diagnostic significance to reach clinical application.[3] Many laboratories have investigated the expression of telomerase activity in tumour versus healthy and non-malignant tissues.[4] Further, investigators have begun to assay telomerase activity in body fluids that might contain tumour cells such as ascitic fluid, pleural

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effusions, urine or bladder washings, oral rinses, sputum, bronchial lavage or washings, and even plasma which can be obtained largely through non-invasive or minimally invasive means.[5] Although cytological examination of cells in these fluids remains the accepted standard for detection of many cancers, in general, the sensitivity is low whereas specificity is high.[2] Hence, we explored the potential of determining sputum telomerase activity as a biomarker for lung cancer.

Human telomerase reverse transcriptase is a ribonucleoprotein that synthesises telomeric sequences, which decrease at each cell division. In cancer cells, its activity is linked to telomere maintenance leading to unlimited cellular proliferation and immortality.[6] Human telomerase is composed of three major subunits, telomerase RNA (hTERC), telomerase reverse transcriptase (hTERT) and telomerase associated protein ($TEP_1$), and their cloning has made it possible to study their expression in various cancers.[7,8] The human telomerase reverse transcriptase (*hTERT*) is an essential component of the holoenzyme complex and the expression level of *hTERT* correlates well with telomerase activity.[9,10] We analysed both *hTERC* and *hTERT* and their correlation to telomerase activity in tissue biopsies of lung cancer.

Upregulation of *c-Myc* expression as well as telomerase activation may contribute to the onset of many types of cancers.[11,12] The cloning and characterisation of *hTERT* gene and its promoters have revealed sites E-boxes for binding of *Myc* oncogene. Evidence that *c-Myc* can induce telomerase activity by transcriptional activation of *TERT*[13] prompted us to examine these associations in lung tumourigenesis.

## 2. Materials and methods

### 2.1. Study design

This study is a prospective analysis of 42 patients with histologically proven lung cancer treated at the Chest Clinic of the Postgraduate Institute of Medical Education and Research, Chandigarh, India. All the cases were newly diagnosed and had not received any form of therapy. The study received formal approval from the Institutional Review Board and in all cases an informed consent was obtained on the proforma prescribed by the institutional ethics committee.

### 2.2. Clinical profile (Table 1)

#### 2.2.1. Lung cancer patients

There were a total of 42 patients, with 39 males and 3 females. Their ages ranged from 45 to 72 years (mean = 58.5). Thirty-two cases were non-small cell lung cancer (NSCLC) upon histology, whereas the rest (10/42) were cases of small cell lung cancer (SCLC). All except four of these patients were smokers. After a thorough clinical examination, each patient underwent CT scan for staging the tumour. On the basis of clinical and radiological findings, there were 37 cases in stage III (37/42), 3 cases in stage IV and 2 cases in stage II.

#### 2.2.2. Negative control group

The control group of 30 patients from whom only sputum was collected included 16 cases of sarcoidosis, 12 cases with a diagnosis of chronic obstructive pulmonary disease and 2 cases of interstitial lung disease.

## 3. Samples

#### 3.0.3. Biopsy

Fibreoptic bronchoscopy was carried out in all patients, the tumour was identified, and at least 3–4 biopsy fragments were obtained. One to two fragments were snap frozen and stored at −80 °C for molecular analysis. The remaining fragments were immersed in buffered formalin and subjected to routine histopathological evaluation. One frozen section from each biopsy was assessed histopathologically to ensure the presence of tumour. All those cases, which were non-representative or contained less than 90% tumour, were not included in the final analysis. The proportion of stromal cells and lymphocytes in the biopsy ranged from 0% to 10% in the sample subjected to molecular analysis. After confirmation of the diagnosis, we proceeded with the molecular evaluation. This included assessment of telomerase activity and the mRNA expression of *hTERC*, *hTERT* and *c-Myc*.

#### 3.0.4. Sputum

Sputum was collected before conducting the bronchoscopy from all patients. The patients were asked to inhale 3% hypertonic saline (3 ml) with the help of an aided nebuliser to induce sputum and were then asked to cough the sputum into a sterile container. Equal amounts of phosphate buffered saline with N-acetyl-L-cysteine (0.25 ml of 20% N-acetyl-L-cysteine in 10 ml of PBS) were added to the sputum sample and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the sediment was stored at −20 °C till further analysis.

### 3.1. Telomerase assay

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) method[15] using the Telo TAGGG telomerase PCR kit (Roche, GmbH, Mannheim, Germany). Thin slices of frozen biopsy samples were homogenised in 200 μl of a detergent based lysis buffer (provided in the kit) and the lysate were incubated on ice for 30 min and centrifuged for 30 min at 12,000*g*. Similarly for the sputum samples the frozen sediment was washed with phosphate buffered saline (PBS) and the pellet was resuspended in 200 μl of the detergent based lysis buffer (provided in the kit). The lysate was then incubated on ice for 30 min and centrifuged for 30 min at 12,000*g*. The protein concentration of supernatant was measured using bicinchonic acid assay (Sigma Aldrich, Inc., Germany). Six micrograms of the cell extracts were incubated with telomerase substrate oligonucleotide (P1-TS) at 25 °C for 30 min followed by 94 °C for 5 min to inactivate the telomerase activity. The extended products were amplified by a polymerase chain reaction (PCR) using Taq polymerase, the P1-TS, P2 primers and nucle-

otides (all of which were present in the master mix provided in the kit). The PCR conditions were 35 cycles of 94 °C for 30 s 52 °C for 30 s, and 72 °C for 90 s followed by a final extension of 72 °C for 10 min. The PCR products were separated on a 12% non-denaturing DNA PAGE (polyacrylamide gel electrophoresis). The gel was fixed in 10% ethanol for 10–15 min and silver staining was carried out in order to visualise the 6 bp pattern typical of telomerase activity. Negative control reactions were performed by heating an aliquot of each cell extract to 85 °C for 10 min and assaying the samples as detailed above. In order to validate this assay, the initial 10 test and control cases were run in duplicate and consistent results were obtained.

### 3.2. *Reverse transcription polymerase chain reaction (RT-PCR)*

Total RNA was isolated using the parallel RNA/DNA isolation kit (Qiagen) from the biopsy tissue. One microgram of template RNA was reverse transcribed to cDNA using 0.5 μg of oligo dT, 15 u AMV reverse transcriptase (Roche, GmbH, Mannheim, Germany), 2 mM each of dNTPs and 20 U RNase inhibitor. The mixture was incubated at 42 °C for 1 h, and subsequently heated for 2 min at 94 °C. The reaction mix was then used for PCR. For all samples a negative control of a similar reaction mix but lacking the reverse transcriptase was also included and used as a template for subsequent PCR.

#### 3.2.1. *Polymerase chain reaction*

PCR was carried out on 2 μl of the cDNA template using 1× PCR buffer (Roche, GmbH, Mannheim, Germany), 2 mM dNTPs each, 100 pmol of unique forward and reverse primers and 1.5 U Taq DNA polymerase in a final volume of 25 μl. The DNA thermal cycler conditions used were 95 °C for 1 min, 55–61 °C for 45 s-1 min and 72 °C for 1 min for 35 cycles followed by a final extension of 72 °C for 8 min.

#### 3.2.2. *Primer sequences*

The primer sequences used were as follows:

β-*Actin* transcript: 5′tct aca atg agc tgc gtg3′ and 5′cct taa tgt cac gca cga3′ (372 bp)

*hTERT* transcript[9]: 5′cgg aag agt gtc tgg agc aa 3′ and 5′gga tga agc gga gtc tgg a3′ (145 bp)

*hTERC* transcript[9]: 5′tct aac cct aac tga gaa ggg cgt ag3′ and 5′gtt tgc tct aga atg aac ggt gga ag3′ (125bp)

*c-Myc* transcript[14]: 5′aag tcc tgc gcc tcg caa-3′ and 5′gct gtg gcc tcc agc aga-3′ (248 bp)

The PCR products were electrophoresed on a 1.5% agarose gel and visualised by ethidium bromide staining under UV illumination.

### 3.3. *Statistical analysis*

Fischer's exact test was applied to determine the association between telomerase and its components and also between telomerase activity in biopsy and sputum samples. The effect of *hTERC* and *c-Myc* on *hTERT* activity was examined by Logistic regression. A probability value of $\leqslant 0.05$ was considered significant.

## 4. Results

### 4.1. *Telomerase activity by TRAP assay in biopsies and paired sputum samples of lung cancer patients*

Telomerase activity was measured in 34 paired biopsy and sputum samples of patients suffering from lung cancer (Table 1) and in 30 control cases of patients suffering from diseases other than lung cancer. A positive telomerase enzyme activity was observed in 29 (85.2%) biopsy tissue samples and 23 (67.6%) sputum samples (Fig. 1). The concordance between telomerase enzyme activity of sputum and biopsy samples of lung cancer patients was found to be statistically significant ($p = 0.002$).

### 4.2. *Telomerase activity by TRAP assay in sputum samples of negative controls*

A total of 30 control patients having lung related pathologies other than lung cancer were evaluated for the telomerase enzyme activity in sputum samples. No telomerase activity was observed in 27 (90%) patients. A very weak telomerase activity as indicated by faint bands on visual inspection of the gel was seen in 3 patients all of whom were cases of sarcoidosis (Fig. 2).

### 4.3. *Sputum telomerase repeat amplification protocol (TRAP) as biomarker of malignancy*

Out of 64 cases analysed comprising 34 positive cases and 30 negative controls, there were 11 false negative and 3 false positive cases. Thus, sputum TRAP assay showed a sensitivity of 67.6%, specificity of 90%, positive predictive value of 88.46% and a negative predictive value of 71% (Table 2).

### 4.4. *mRNA expression of the subunit genes hTERT, hTERC* of telomerase and c-Myc in tissue biopsy samples of lung cancer patients (Table 1)

Expression of the *hTERT*, *hTERC* and *c-Myc* transcripts was observed in 95.2%, 92.8% and 80.9% of the tumour tissues, respectively (Fig. 3), and positive telomerase expression was found in 36 (85.7%) cases out of a total of 42 patients (Table 2). We looked for an association of each of these parameters with the telomerase activity using the Fischer's exact test. The telomerase activity correlated with *hTERT* expression ($p = 0.002$) but not with the *hTERC* ($p = 1.000$) or *c-Myc* transcript expression ($p = .088$).

The relation of *c-Myc* expression on *hTERT* was examined and found to be not significant by Fisher's exact test ($p = 0.080$) and of borderline significance by logistic regression analysis ($p = 0.053$). The *hTERT* expression was independent of *hTERC* expression as well ($p = 0.909$).

## 5. Discussion

Over the past few years numerous studies have shown that telomerase is expressed and active in various types of tumours.[4] Telomerase activity has been measured as a biomarker for the detection of cancer in various clinical

**Table 1 – Clinical profile, telomerase, *hTERC*, *hTERT* and *c-Myc* in biopsy samples and telomerase in paired sputum samples from subjects with lung cancer**

| Case No. | Age/sex | Smoking History | Histology type/stage | RT-PCR | | | Telomerase (TRAP assay) | |
|---|---|---|---|---|---|---|---|---|
| | | | | *hTERC* | *hTERT* | c-Myc | Biopsy | Sputum |
| 1 | 60/M | + | SqCC/III | + | + | – | + | + |
| 2 | 55/F | – | SqCC/III | + | + | – | + | – |
| 3 | 62/M | + | SqCC/III | + | + | + | + | – |
| 4 | 65/M | + | SqCC/II | + | + | + | + | + |
| 5 | 62/M | + | SqCC/III | + | + | + | + | + |
| 6 | 72/F | + | SqCC/III | – | + | – | – | – |
| 7 | 65/M | + | SqCC/III | + | + | + | + | + |
| 8 | 70/M | + | SqCC/III | + | + | + | + | + |
| 9 | 46/M | – | SqCC/III | + | + | + | + | + |
| 10 | 55/M | – | SqCC/III | + | + | + | – | – |
| 11 | 46/M | + | SqCC/III | + | + | + | + | + |
| 12 | 52/M | + | SqCC/III | + | + | + | + | + |
| 13 | 50/M | + | SqCC/III | + | + | + | + | + |
| 14 | 55/M | + | SqCC/III | + | + | + | + | + |
| 15 | 55/M | + | SqCC/III | + | + | + | + | + |
| 16 | 54/M | + | SqCC/III | + | + | + | + | + |
| 17 | 52/M | + | SqCC/III | + | + | + | + | + |
| 18 | 41/M | – | SqCC/III | + | + | – | + | + |
| 19 | 58/M | + | SqCC/III | + | + | + | + | + |
| 20 | 50/M | + | SqCC/III | + | + | + | + | + |
| 21 | 65/M | + | SqCC/II | + | + | + | + | + |
| 22 | 55/M | + | SqCC/III | + | + | + | – | |
| 23 | 40/M | + | SqCC/III | + | + | + | + | + |
| 24 | 55/F | + | SqCC/III | + | + | + | + | + |
| 25 | 60/M | + | SqCC/III | + | + | + | + | ND |
| 26 | 52/M | + | SqCC/III | + | + | + | + | – |
| 27 | 70/M | + | SqCC/III | + | – | - | – | – |
| 28 | 38/M | + | SqCC/III | + | + | + | + | – |
| 29 | 52/M | + | SqCC/III | + | – | + | – | ND |
| 30 | 62/M | + | SqCC/III | + | + | – | + | ND |
| 31 | 52/M | + | SqCC/III | + | + | + | + | ND |
| 32 | 60/M | + | SqCC/III | + | + | + | + | ND |
| 33 | 64/M | + | SCLC/III | + | – | – | – | – |
| 34 | 45/M | + | SCLC/III | + | + | + | + | – |
| 35 | 65/M | + | SCLC/IV | + | + | + | + | + |
| 36 | 63/M | + | SCLC/III | + | + | – | + | + |
| 37 | 62/M | + | SCLC/III | – | + | + | + | + |
| 38 | 58/M | + | SCLC/IV | + | + | + | + | + |
| 39 | 62/M | + | SCLC/III | + | + | + | + | – |
| 40 | 50/M | + | SCLC/III | + | + | + | + | ND |
| 41 | 55/M | + | SCLC/III | + | + | + | + | ND |
| 42 | 52/M | + | SCLC/IV | + | + | + | + | ND |

ND, not done; SqCC, squamous cell carcinoma; SCLC, small Cell lung cancer.

specimens obtained by non-invasive or minimally invasive methods such as urine, cervical scrapings and fine needle aspirates.[16–19] For lung cancer detection, this technique has been applied to bronchial washings and sputum.[20,21] In the present study, we address this issue and also include an equivalent number of control patients in order to critically evaluate the sensitivity and specificity of using telomerase activity as a non-invasive biomarker in sputum.

The sensitivity of using telomerase as a biomarker was 67.6% and specificity was 90% in our study. A comparison of the present study with previous studies on the evaluation of telomerase in respiratory secretions is shown in Table 3. It may be noted that whereas many studies have evaluated telomerase activity in bronchial washings or lavage,[20–24] ours is the second study to perform it in sputum. Bronchial washings or lavage are obtained by bronchoscopy, an invasive procedure that is not without the attendant risks and cannot be applied for screening purposes. On the other hand, sputum is the only respiratory secretion that may be examined for lung cancer screening. In the previous study on sputum by Sen and colleagues where only 10 negative control cases were examined, the sensitivity was 80% and specificity was 100%. We have examined a large number of 'negative control' cases, which included cases of interstitial lung disease, sarcoidosis and chronic obstructive airway disease. Hence, our estimate of specificity is more robust and likely more representative of the test characteristics. The three false positive TRAP assays were from subjects with sarcoidosis, and the weak telo-

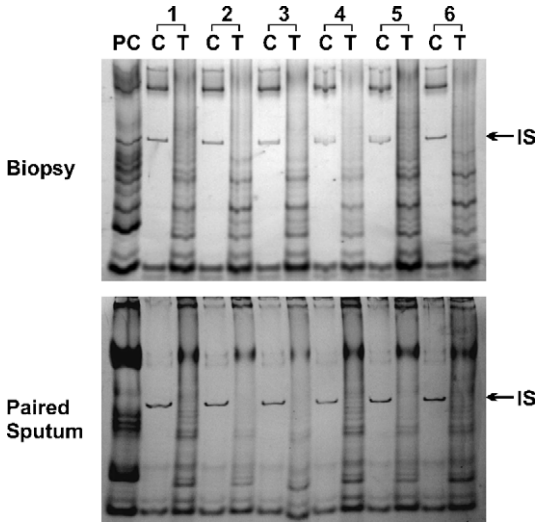


**Fig. 1 – TRAP assay in biopsies and paired sputum samples of lung cancer. IS, internal standard; PC, positive control; T, test; C, heat-inactivated control. All six cases show telomerase activity in paired biopsy and sputum samples.**

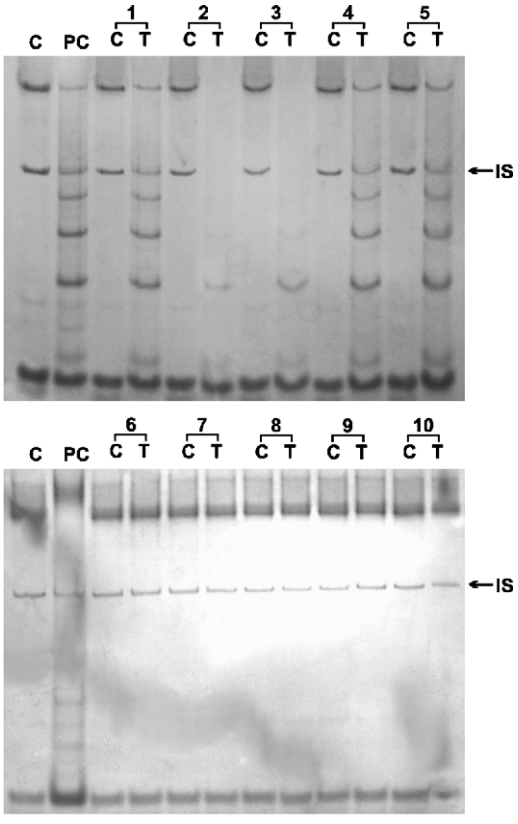


**Fig. 2 – Sputum telomerase activity in subjects without evidence of lung cancer. PC, positive control, T, test; C, heat-inactivated control. In subjects 1, 4 and 5, weak telomerase activity is noted whereas it is absent in subjects 2, 3, 6–10.**

merase positivity was most likely derived from lymphocyte contamination. Arai and colleagues have reported positivity for telomerase in bronchoalveolar lavage fluid samples in

**Table 2 – Sputum telomerase repeat amplification protocol (TRAP) assay as biomarker of malignancy**

| | Sputum TRAP | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Lung cancer present | 23 (TP) | 11 (FN) | 34 |
| No evidence of lung cancer | 3 (FP) | 27 (TN) | 30 |
| Sensitivity | | | 67.6% |
| Specificity | | | 90% |
| Negative predictive value | | | 88.46% |
| Positive predictive value | | | 71% |

TP, true positive; FN, false negative; TN, true negative; FP, false positive.

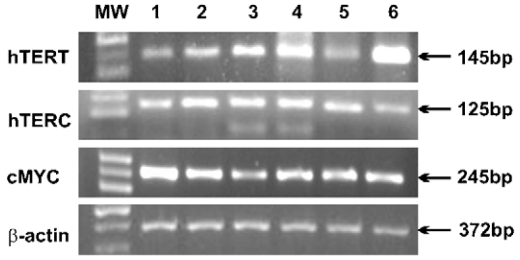


**Fig. 3 – Expression of transcripts of *hTERT*, *hTERC* and *c-Myc* in biopsies of lung cancer. MW, 50 bp ladder and lanes 1–6 represent six cases.**

two cases of sarcoidosis and one case of pulmonary tuberculosis. Telomerase positivity in non-neoplastic inflammatory conditions has also been reported previously in cases of effusions with lymphocytic infiltration[5] and in lymphocytic thyroiditis.[19] On the other hand, numerous inflammatory cells comprising polymorphs, histiocytes and lymphocytes were also present in those sputum samples, which tested negative for telomerase activity. Hence, it is possible that mere presence of inflammatory cells does not result in a positive telomerase assay and that in some patients with sarcoidosis, there is a mild telomerase activation resulting in TRAP assay positivity.

Cytological examination for malignant cells was successful in the detection in only four out of 30 cases proven to have lung cancer. Histologically, there were 32 squamous cell carcinomas and 10 small cell carcinomas. The very low sensitivity of cytology in our set up could be attributed to the fact that we did not use any method of sputum concentration. Thus although it has been suggested that TRAP assay results may be best interpreted in conjunction with cytological examination,[5] but the performances of combined cytology/telomerase screening algorithms in different laboratories have not been defined. In view of the vastly greater sensitivity over conventional sputum cytology, we believe that telomerase testing may be routinely applied to sputum samples. Early detection of lung cancer is a challenge[2] and evaluation of sputum is the only non-invasive means available to us. In view of the lower than desired sensitivity, sputum telomerase detection may be combined with other biomarkers for the early detection of lung cancer. Recently, methylation profiling of *p16INK4A*, RARβ,[22] *RASSF1A* and *3-OST-2* genes[25] and detection of loss

**Table 3 – Telomerase assay versus conventional cytology for detection of malignancy in respiratory secretions**

| Study | No. of cases | Sample | TRAP assay | | Cytology | |
|---|---|---|---|---|---|---|
| | | | Sens % | Spf% | Sens% | Spf% |
| Yahata et al.[20] | 22 | Br wash | 82 | NA | 41 | NA |
| Arai et al.[22] | 37 | Br wash | 78.4 | NA | 64.7 | NA |
| Xinarinanos et al.[23] | 36 | Br lavage | 70 | NA | 43 | NA |
| Sen et al.[21a] | 42 | Br wash | 68.4 | NA | NA | NA |
| Dikmen et al.[24] | 29 | Br wash | 72.7 | 85.7 | 65.5 | NA |
| Sen et al.[21a] | 42 | Sputum | 81.6 | 100 | 39.5 | NA |
| Present study | 64 | Sputum | 67.6 | 90 | 11.7 | 100 |

Br, bronchial; Sens, sensitivity; Spf, specificity; NA, not available.
a Same study.

of heterozygosity using microsatellite polymorphic markers[26] in sputum have shown promising results.

*hTERT* believed to be the rate limiting component amongst the three major subunits comprising the human telomerase complex. The RNA component of human telomerase (*h-TERC*) provides the template for telomere repeat synthesis. Our data confirm that *hTERC* expression is present in the majority of tumour lung tissues; however, the telomerase activity correlated only with *hTERT* but not with *hTERC*, which is in agreement with previous studies.[27–29] This indicates that hTERT is the principal regulator of telomerase activity in lung cancer.

*c-Myc* is believed to be an important regulator of *hTERT* expression in several cancers and its relation has been recently examined in lung cancer tissues.[30,31] In non-small cell lung cancer, a highly significant correlation was observed between the expression of *hTERT* and *c-Myc* in one study ($p < 0.001$),[30] whereas in another study only a weak correlation was observed ($p = 0.042$).[31] In our series the relationship between *c-Myc* expression and *hTERT* is only borderline ($p = 0.053$) and is in broad agreement with the latter report. The discrepant cases included one case positive for *c-Myc* but negative for *TERT* and five cases negative for *c-Myc* but positive for *TERT* expression. Thus, it is possible that in a proportion of advanced lung cancer cases, factors other than *c-Myc* might have a role in regulating *TERT* and further studies are clearly indicated.

To conclude, sputum telomerase assay holds potential to be used in a panel as one of the biomarkers of lung cancer. Prospective clinical trials need to be designed to critically evaluate its role in early diagnosis of lung cancer. *hTERT* is the principal regulator of telomerase activity and in a proportion of lung cancer cases, *hTERT* regulation may be independent of c-Myc oncogene expression.

## Conflict of interest statement

We the authors, Teena Pasrija, Radhika Srinivasan, Digambar Behera, Siddhartha Majumdar, declare that we have no personal, financial or any kind of relationship with any company or person that may inappropriately influence (bias) the work. We hereby declare that there is no conflict of interest. Further, this article has not been published earlier and is not under consideration in any other journal.

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