Egyptian Journal of Bronchology

Vol 2, No 2, December, 2008

ORIGINAL ARTICLE

AMPLIFICATION OF HUMAN TELOMERASE REVERSE TRANSCRIPTASE GENE AS A DIAGNOSTIC MARKER IN LUNG CANCER

By
Ibtesam Farid,[1] Khadiga Abu Gabal,[2] Mohamed El-Batanony,[3] Mahmoud El-Batanouny,[4] Manal Kamal,[1] Yasser Hassan[1]

[1]Clinical and Chemical Department, Faculty of Medicine, Cairo University. [2]Clinical and Chemical Department, Faculty of Medicine, Beni-Suef University. [3]Occupational Diseases Department, Faculty of Medicine, Cairo University. [4]Chest Diseases Department, Faculty of Medicine, Beni-Suef University.

Purpose: ***Analysis of circulating DNA or RNA in plasma can provide a useful marker for earlier lung cancer detection. This study was designed to assess the sensitivity and specificity of a quantitative molecular assay of circulating RNA to identify patients with lung cancer with different grades.***

Materials and Methods: ***The amount of plasma RNA was determined through the use of real-time quantitative polymerase chain reaction (PCR) amplification of the human telomerase reverse transcriptase gene (hTERT) in 19 non–small-cell lung cancer patients and 10 age and sex matched controls. Performance of the assay was calculated through the receiver operating characteristic (ROC) curve.***

Results: ***The hTERT mRNA ratio in cancer lung patients showed a mean of 196.34±307.23 which was higher compared to that of the controls 1.24±0.80; this difference was statistically highly significant where P<0.01. The median concentration of circulating plasma RNA in patients was higher than the value detected in controls (71.70 v 1.149 ratio). Plasma RNA was a strong risk factor for lung cancer; concentrations in the patients were associated with a 62-fold higher risk than were those in the controls. The point of the best cut-off value was at 2.24 where sensitivity was 73.7 % and specificity was 90 %. The area under the ROC curve was 0.704.***

Conclusion: ***This study shows that higher levels of free circulating RNA can be detected in patients with lung cancer compared with disease-free heavy smokers by a PCR assay, and suggests a new, noninvasive approach for early detection of lung cancer. Levels of plasma RNA is recommended to be measured as it could also identify higher-risk individuals for lung cancer screening and chemoprevention trials.***

INTRODUCTION

Lung cancer is the leading cause of cancer mortality throughout the world and is the cause of more than 1 million annual deaths.[27] It has one of

the lowest survival outcomes of any cancer because over two-thirds of patients are diagnosed at a late stage when curative treatment is not possible. In Europe, only 10% can be cured and can benefit from long-term survival because of the absence of early detection plans, the frequency of metastases at diagnosis, and poor responsiveness to chemotherapy. However, survival of patients undergoing lung resection for small intrapulmonary cancers is greater than 80%.(26) Despite major potential for prevention, complete eradication of smoking has proven difficult, and the risk of cancer remains high in former smokers. As a consequence, there is a need to develop new tests that may facilitate earlier diagnosis and more effective treatment. Low-dose spiral computed tomography (CT) scan of the chest has been effective in detecting small tumors, with a high proportion of resectable (96%) and stage I (80%) disease.(11) Conversely, increased knowledge of molecular pathogenesis of lung cancer offers a basis for the use of molecular markers in biologic fluids for early detection as well as identification of higher-risk smokers.

The ability of CT scans to detect early stage lung cancer is undisputable. What is unresolved is the ability of spiral CT screening to affect lung cancer-related mortality. Genomic and proteomic approaches promise to complement the ability of spiral CT to detect early lung cancer in the next few years. Currently, the decision to screen for lung cancer should involve a careful discussion with the individuals involved about the potential advantages, costs, and drawbacks of the approach.(8)

The report(2) that tumor-derived RNA is detectable in cell-free serum of cancer patients has opened up a new molecular approach for the early detection and follow-up of cancer. Because telomerase activity is the most general molecular marker for the identification of human cancer, the analysis of telomerase mRNA expression in serum is attractive as a potential tumor marker. Telomerase is a RNA-dependent DNA polymerase that stabilizes telomeres and allows cells to avoid the senescence checkpoint. Several lines of evidence indicate that telomerase is involved in the attainment of immortality in cancer cells and may therefore contribute to tumorigenesis and neoplastic progression.(15) Overall, telomerase activity has been found in a high percentage of human tumors (>85%), whereas only a limited fraction of cancers maintains telomeres through an alternative lengthening of telomere (ALT) recombination mechanism. The core enzyme consists of a RNA component (hTR) that provides the template for the de novo synthesis of telomeric DNA and a catalytic subunit [human telomerase reverse transcriptase (hTERT)] with reverse transcriptase activity.(20) Different genes encode for the various components of the human telomerase complex. A good correlation between telomerase reverse transcriptase (hTERT) expression and telomerase activity has been reported, and quantitative studies in serum or plasma have been performed to date. The present study reports the development of a quantitative assay for the measurement of hTERT expression in the plasma of cancer patients based on real-time quantitative RT-PCR (qRT-PCR) normalized to the amount of RNA input.(2) As shown in (Fig. 1), the outcome of telomere dysfunction includes the generation of a persistent DNA damage signal. The fate of cells that experience this signal, genomic instability, senescence (permanent arrest of cell growth) or apoptosis (programmed cell death), depends critically on p53.This cell fate subsequently influences the organismal phenotypes of cancer and aging.(28)

Common genetic alterations in lung carcinogenesis include allelic loss and instability at loci on 3p (fragile histidine triad [FHIT]), 9p (p16INK4A), and 17p (p53);(29) aberrant promoter methylation of p16INK4A, APC (adenomatous polyposis of the colon), and other tumor suppressor genes;(22,43) and Kirsten rat sarcoma (KRAS)(19) and p53 mutations.(10,34,37) Detection of these changes in DNA derived from body fluids such as sputum, bronchial brush and lavage, and plasma or serum of lung cancer patients and chronic smokers has been proposed by several authors(33,36,39) as a potential diagnostic tool. However, the sensitivity and specificity of detection assays in these biologic

samples have been limited by the low frequency of alterations of each specific gene, relative low-sensitivity of used methodologies, and choice of appropriate markers.

Analysis of circulating DNA in plasma is a promising noninvasive diagnostic tool, requiring only a limited blood sample. The intent of this study was to set up a relatively simple blood test on the basis of a single marker, to be potentially applicable to large-scale trials for early lung cancer detection. In a previous report, using a DNA colorimetric assay, some authers have shown a higher plasma DNA concentration in lung cancer patients than in controls, regardless of tumor stage, suggesting that plasma DNA was an early event in lung carcinogenesis.[35] Because telomerase activity is the most general molecular marker for the identification of human cancer, the analysis of telomerase mRNA expression in serum is attractive as a potential tumor marker.[2] To measure with greater accuracy the amount of free circulating DNA, a quantification approach based on real-time quantitative polymerase chain reaction (PCR) was developed.[18] A single copy gene, the amplification of which is specific and robust, represents the ideal target for DNA-based quantitative real-time PCR assay. For this study, we selected an assay designed for the human telomerase reverse transcriptase (hTERT) genomic sequence that performed consistently in preliminary experiments. Amplification of hTERT was therefore used as a marker of the total amount of DNA present in plasma samples. We considered that hTERT expression and telomerase activity have been reported as prognostic factors in stage I non–small-cell lung cancer (NSCLC) patients.[40] However, our working hypothesis was not based on the evaluation of hTERT expression at the transcriptional level as a tumor-associated marker, but was based instead on the use of a single copy gene such as hTERT as an indicator of the global amount of circulating DNA. The sensitivity and the specificity of the test were validated in a case-control study of 19 cancer lung individuals and in a control group of age and sex matched persons.

METHODS AND SUBJECTS

Patients and Control Series: We evaluated 19 newly diagnosed patients with NSCLC, 14 males and 5 females, who were selected from the National Institute of Oncology and Kasr Al-Aini, Cairo University and not previously treated with chemoradiotherapy. Their age ranged between 26-72 years with a mean of 53.7±11.8 years. All patients had primary cancers and were receiving first treatment; no patients with disease relapse or follow-up were included.

Ten age and sex matched individuals with normal laboratory and radiological findings were included as a control group. They were 8 males and 2 females. Their age ranged between 28-70 years with a mean of 55.1±13.2 years.

HTERT mRNA was measured in all patient samples in comparison with normal healthy controls.

The following four steps were done:

(I) Sample collection.

(II) RNA extraction.

(III) Complementary DNA formation.

(IV) Real time PCR quantification.

Sample Collection:

A sample (3 ml) of peripheral blood was collected in tubes containing EDTA, from patients before surgery and from controls. Plasma separation and RNA extraction were performed[35] and stored at -70°C.

RNA extraction:

The kit name was QIAmp® RNA Blood Mini Kits from QIAGEN,[14] Catalog No. 52304. Extraction was done according to a published, modified protocol adapted to isolation of RNA from plasma samples.[30]

Complementary DNA formation:

The kit name was High-Capacity cDNA Archive Kit.[6] Applied Biosystems Part No. 4322171. Procedures were done to prepare the 2XRT master mix (per 20-ul reaction) and to perform reverse

transcription as tubes were placed into the programmed thermal cycler then cDNA was stored at –20oC.

About Real-Time PCR Assays:

Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e. in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.[36]

RNA Quantification in Plasma:

To quantify the circulating RNA in plasma, we used a real-time quantitative PCR approach based on the 5' nucleotide method. This methodology is based on continuous monitoring of a progressive fluorogenic PCR by an optical system. The PCR system uses two amplification primers and an additional amplicon-specific and fluorogenic hybridization probe, the target sequence of which is located within the amplicon. The probe is labeled with two fluorescent dyes. One serves as a reporter on the 5' end (VIC dye; Applied Biosystems, Foster City, CA). The emission spectrum of the dye is quenched by a second fluorescent dye at the 3' end (TAMRA; Applied Biosystems). When amplification occurs, the 5' to 3' exonuclease activity of the AmpliTaq (Applied Biosystems) DNA polymerase cleaves the reporter from the probe during the extension phase, thus releasing it from the quencher.[5,12] The resulting increase in fluorescent emission of the reporter dye is monitored during the PCR process.

Primers and probes were designed to specifically amplify the ubiquitous gene of interest, the hTERT single copy gene. The amplicon size of the hTERT gene was 98 bp, Applied Biosystems part number: 4331l82. The sequences of the primers and of the probe were the following: the forward primer, hTERT–Fw: 5'-ACCGTCTGCGTGAGGAGATC-3'; the reverse primer, hTERT –Rv: 5'-CCGGTAGAAAAAGAGCCTGTTC-3'; the TaqMan probe, hTERT–probe:5'- F A MTGT A CGTCGTCG A GCTGCTC A GGTCTTT-3'-TAMRA.[5]

Fluorogenic PCRs were carried out in a reaction volume of 25 uL on a GeneAmp Stratagene Mx3005 sequence detection system. Fluorogenic probe and primers were custom synthesized by Applied Biosystems. Thermal cycling was initiated with a first denaturation step of 50°C for 2 minutes and then 95°C for 10 minutes. The thermal profile for the PCR was 95°C for 15 seconds and 60°C for 1 minute. Data obtained during 50 cycles of amplification were analyzed.

All of the data were analyzed using the Sequence Detection System software (Stratagene Mx3005) to interpolate the standard amplification curve of DNA at a known quantity with amplification cycle threshold of the unknown target sample, thus obtaining the relative amount of DNA in the experimental sample. Interpretation of results was done using the comparative CT method for Relative Quantitation according to this Arithmetic Formulas:

$2^{-\Delta\Delta CT}$

Arithmetic Formulas: The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by: $2^{-\Delta\Delta CT}$

Where's CT = Threshold cycle.

$\Delta CT = CTt - CTr$

Where's ΔCT = the difference in threshold cycles for target and reference.

$\Delta\Delta CT = \Delta CTcases - \Delta CTcalibrators$

A relative quantitation assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample). The comparative CT method requires an endogenous control, which is a gene target used to normalize quantitative PCR results. The endogenous control should consistently express in all samples

and the expression level should not be altered by any experimental condition. The hTERT mRNA expression in a sample was determined by substracting the cycle threshold of the reference gene from the target gene to get the normalized amount of the hTERT mRNA, then comparing this value to that is of the calibrator.

Terms Used

CT = Threshold cycle.

The CT is the cycle number at which the fluorescence generated within a reaction crosses the threshold line. CT values are logarithmic and are used either directly (comparative CT method) or indirectly (interpolation to standard curves to create linear values) for quantitative analyses.

Statistical Methods: The distribution of DNA values revealed a departure from normality that was mitigated using a logarithmic transformation. The log of the concentration was used for testing purposes. SPSS statistical software package, V. 13, Echosoft Corp., USA, 2003 was used for data analysis. Wilcoxon Rank Sum test for comparison between two independant groups for non-parametric data. The probability of error at 0.05 was considered significant while at 0.01 and 0.001 are highly significant.

A receiver operating characteristic curve (ROC) was developed to evaluate the diagnostic performance of plasma DNA concentrations. Each unique DNA value was used as a cutpoint to calculate sensitivity and specificity values defining the curve and the area under the curve (AUC) and 95% CI for the area.

RESULTS

The amplification plots of fluorescence intensity against the PCR cycle from plasma samples of lung cancer patients and matched controls re shown in (Fig. 2).

The hTERT mRNA ratios in plasma samples of normal controls ranged between 0.398 and 2.521 with a mean of 1.2477±0.8044 while the hTERT mRNA ratios in plasma samples of cancer lung patients ranged between 0.282 to 1195.9 with a mean of 196.3432±307.2345 s shown in Table l.

A comparison between mean±SD values of hTERT mRNA ratios in cancer lung patients and controls, hTERT mRNA ratio in cancer lung patients had a mean of 196.3432±307.2345 which was higher than that of the controls (mean 1.2477±0.8044). The difference was statistically highly significant ($P<0.01$) s shown in Table 2 and (Fig 3).

The best cut-off value was 2.24 at which the true negative was 9 out of 10 control's samples; the true positive were 14 out of 19 cancer patients' samples; the false negative were only 5 samples. At this cut-off the sensitivity and the specificity were 73.7 % and 90.0 % respectively; the negative predictive and the positive predictive values were 64.3 %& 93.3 % respectively with efficacy of 79.3 % as shown in table 3 and (Fig 4).

The median values and the CI of hTERT mRNA ratio of patients and controls are shown in table 4. The median value and the CI of patient's ratio were 71.7 & 148.0823 respectively while for controls they were 1.14950 & 0.545 respectively.

Table 1. The hTERT mRNA ratio in plasma samples of normal control and cancer lung patients.

	Controls	Cancer lung patients
Age (yrs)	55.1±13.2	53.7±11.88
tΔC	23.794±1.05619	17.96±6.1
ΔΔCt	8.3317±1.056	-4.726±7.00
Ratio	1.247±0.8044	196.34±307.23

Table 2. Comparison between hTERT mRNA ratio between cancer lung patients and controls.

Item	Mean±SD	Z value	P value	Sig.
Controls ratio	1.247±0.8044	-2.84511	0.00444	<0.01 highly significance
Patients ratio	196.34±307.234			

Table 3. At the point of best cut-off value (2.24).

	Cases	Controls	Sp.	Sn.	P-	P+	Eff.
Test+ve	14	1					
Test-ve	5	9	90.0	73.7	64.3	93.3	79.3
Total number	19	10					

Table 4. The CI and the median values of patients and control ratios.

Item	Control's ratio	Patient's ratio
Confidence interval 95% lower	0.67223	48.260863
Confidence interval 95% upper	1.82317	344.425485
Median	1.14950	71.7
CI	0.54508331	148.082311

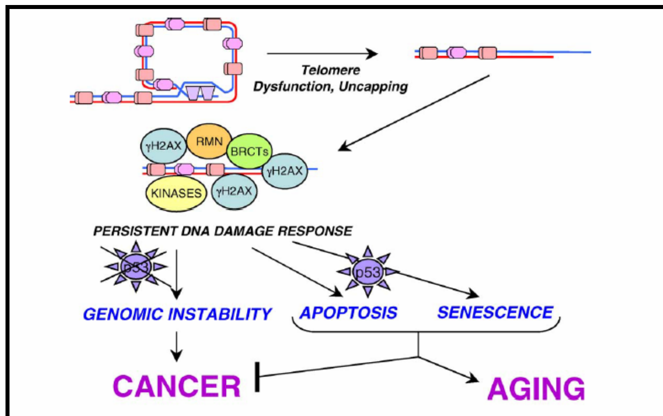


Fig 1. The outcome of telomere dysfunction includes the generation of a persistent DNA damage signal.[28]

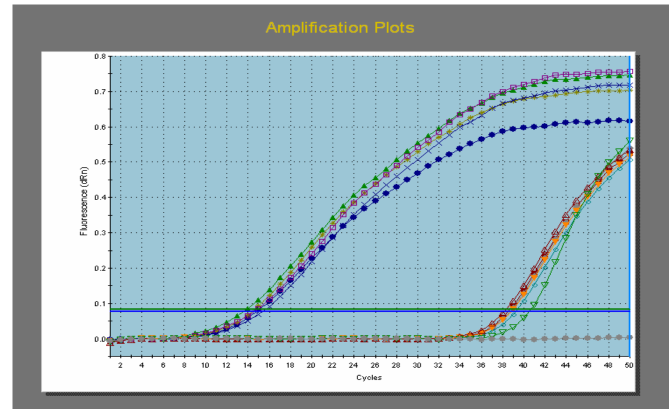


Fig 2. It shows amplification plots of fluorescence intensity against the PCR cycle from plasma samples of lung cancer patients and matched controls. Each plot corresponds to the initial target DNA quantity present in the sample. Calculation of the amounts of DNA is based on the cycle number, where fluorescence of each reaction passes the cycle threshold, which is set to the geometric phase of the amplification above the background. The x-axis denotes the cycle number of a quantitative PCR reaction. The y-axis denotes the log of fluorescence intensity over the background (ΔRn). The relative amount of DNA is much higher in patient samples (left plots) compared with those of controls (right plots). The amplification curves that are shifted to the right, representing reduced target DNA quantity, clearly discriminate controls from lung cancer patients.

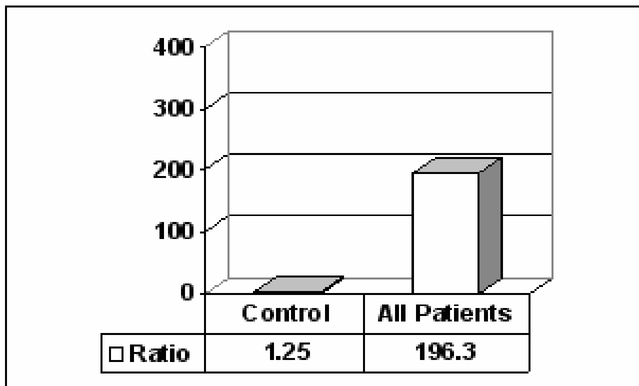
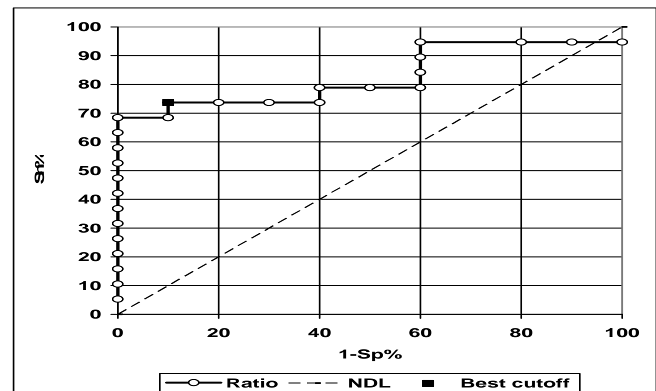


Fig 3. Comparison between controls and patients as regards mean values of ratios.



AUC	
0.704	Ratio

Fig 4. ROC curve analysis showing the diagnostic performance of ratio for discriminating patients with cancer lung from controls.

DISCUSSION

Evaluation of tumor-specific circulating DNA in plasma/serum is a promising noninvasive diagnostic and prognostic tool requiring only a blood sample, which circumvents the logistic difficulties associated with the use of invasive procedures for serial tumor samplings during screening of lung cancer. In healthy persons, it can be presumed that circulating DNA originates from lymphocytes or other nucleated cells. Its origin in malignancies is still unknown. Many mechanisms have been postulated the release of DNA into the circulation by tumor–host interactions. One of the mechanisms may involve lysis of cancer cells shed into the circulation by micrometastases.(25) Cell necrosis may be another possible mechanism because higher amounts of DNA have been found in the plasma of patients with large or advanced metastatic tumors.(16) Apoptosis has also been advanced as a possible source of circulating cell-free DNA on the basis that circulating DNA often gives a ladder pattern on electrophoresis similar to that shown by apoptotic cells.(32) Spontaneous, active release of DNA by tumors is another possibility. The possibility that proliferating cancer cells release DNA into the circulation cannot be ignored because activated lymphocytes have been found to release DNA under in vitro conditions. This could explain the presence of very low concentrations of cell-free DNA in small populations of cancer patients. In these cases, the cancer may have been relatively quiescent at the time of specimen collection.(25)

Telomerase has been the focus of intense study because of its aberrant up-regulation in the majority of cancer cell lines.(9,42) The report that tumor-derived RNA is detectable in cell-free serum of cancer patients has opened up a new molecular approach for the early detection and follow-up of cancer. Because telomerase activity is the most general molecular marker for the identification of human cancer, the analysis of telomerase mRNA expression in serum is attractive as a potential tumor marker.(2,3)

Several reports have described the detection of circulating, cancer-related RNA molecules in serum or plasma from cancer patients.(7) Despite substantial advances in cancer diagnostics, the search persists for simple and cost-effective diagnostic tests. Many studies have explored the possibility of using circulating tumor cells for detection or monitoring of cancer.(17,21,24,38,41) Although circulating tumor cells are frequently detectable in blood from patients with advanced stages of the disease, these researches have been unable to demonstrate reliable utility for early cancer detection or recurrence monitoring.

By using a simple colorimetric assay in a representative series of lung cancer patients and controls, some studies have demonstrated that a quantitative plasma DNA test is a valuable diagnostic tool to discriminate patients from healthy individuals and to detect early recurrence during follow-up. A recent study performed in a group of miscellaneous tumors confirmed these results by using a fluorometric assay and supported a digital single nucleotide polymorphism analysis of allelic imbalances as a sensitive and specific tool for ovarian cancer screening.(1)

We reported here the results of case-control study for validation of free circulating RNA in plasma as a potential lung cancer diagnostic marker. Our results show that real-time quantitative PCR assay, using the hTERT gene as a target sequence for quantification of circulating RNA in plasma, has high sensitivity and specificity, as estimated by AUC ROC curves, by analyzing values as selected cut-points. Furthermore, median concentration in patients (71.7) was almost 62 x the concentration detected in controls (1.14950). Although the highest sum of sensitivity (73.7%), specificity (90%), positive predictive value (93.3%), and negative predictive value (64.3%) was obtained with a DNA concentration value of 2.24, the CI around these diagnostic indicators overlap with those of adjacent concentrations. The selection of the optimal cutpoint will therefore have to acknowledge this variability. The value of 2.24 is the only cutpoint shown with sensitivity that does not overlap with that of other cutpoints. This was

agreed with other researchers[36] who reported that the magnitude proves the strong association between plasma DNA concentration and NSCLC risk, despite wide confidence limits. One important aspect of the quantitative analysis was the ability to follow longitudinal changes after cancer resection. They represented their data available on cancer patients showed a rapid decrease of circulating DNA values after lung resection. Conversely, no decreasing or increasing levels of plasma DNA identified individuals with recurrence of their disease suggesting that quantification of plasma DNA might represent a novel approach to monitor surgical patients or assess treatment efficacy after chemoradiotherapy. In accordance with these results, previous studies reported elevated levels in 78 out of 100 (78 %) lung cancer patient. Miura et al.,[23] reported that in 112 patients with cancer lung, the sensitivity and specificity for hTERT mRNA as a diagnostic marker was 89.0% and 72.7% respectively.

Our results suggest that Real-time RT-PCR quantification is a specific, sensitive, and rapid method that can elucidate the biological role of TERT in tumor development. We found increased amounts of circulating plasma RNA in samples from any stage and tumor size. This is particularly relevant for small lesions, the systematic detection of which could help reduce lung cancer morbidity and mortality which was agreed with other scientists.[9,20,42] In the present study, plasma hTERT mRNA was measured by quantitative RT-PCR. Also, some previous studies [5,31,36] reported using plasma samples while other studies reported using serum samples.[2,23] Jang et al.,[13] used peripheral blood lymphocytes. They based their study on the relative telomere length in peripheral blood lymphocytes using quantitative polymerase chain reaction. In the present study, the mRNA of the catalytic subunite of the telomerase enzyme (hTERT mRNA) was used as a target sequence for quantification, Miura et al.,[23] & Sirera et al.,[31] also used the same target. Sozzi et al.,[36] used hTERT gene itself as a target sequence for quantification of circulating DNA. However Dasi et al.,[4] reported elevated hTERT expression in plasma samples from colorectal cancer and follicular lymphoma, also reported[5] elevation of the same marker in prostate cancer (PCa) patients. It has also been reported[2] that hTERT was found in the serum samples of 4 out of 16 (25%) patients with breast cancer; they also found elevated level of the hTERC subunit of the enzyme in 5 out of 18 (28%) serum samples. This may give an impression that hTERT is a general cancer marker.

CONCLUSION& RECOMMENDATION

The results showed that measuring hTERT mRNA was highly discriminating between healthy subjects and cancer lung patients and strongly supports the idea that a valuable diagnostic test for cancer might be developed using this genetic marker in plasma.

Future studies involving a large number of cases, serial blood analysis, correlation with different markers and histopathological types & TNM staging of the disease may allow a more detailed assessment of the predictive ability of this assay.

Long-term follow-up of hTERT mRNA elevation to monitor surgical patients or assess treatment efficacy after chemoradiotherapy of patients receiving therapy is highly recommended.

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