# Prognostic value of hTERT mRNA expression in surgical samples of lung cancer patients: the European Early Lung Cancer Project

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**Abstract.** Lung cancer is the most important cause of cancer-related mortality. Resectability and eligibility for treatment with adjuvant chemotherapy is determined by staging according to the TNM classification. Other determinants of tumour behaviour that predict disease outcome, such as molecular markers, may improve decision-making. Activation of the gene encoding human telomerase reverse transcriptase (hTERT) is implicated in the pathogenesis of lung cancer, and consequently detection of hTERT mRNA might have prognostic value for patients with early stage lung cancer. A cohort of patients who underwent a complete resection for early stage lung cancer was recruited as part of the European Early Lung Cancer (EUELC) project. In 166 patients expression of hTERT mRNA was determined in tumour tissue by quantitative real-time RT-PCR and related to that of a house-keeping gene (PBGD). Of a subgroup of 130 patients tumour-distant normal tissue was additionally available for hTERT mRNA analysis. The correlation between hTERT levels of surgical samples and disease-free survival was determined using a Fine and Gray hazard model. Although hTERT mRNA positivity in tumour tissue was significantly associated with clinical stage (Fisher's exact test p=0.016), neither hTERT mRNA detectability nor hTERT mRNA levels in tumour tissue were associated with clinical outcome. Conversely, hTERT positivity in adjacent normal samples was associated with progressive disease, 28% of patients with progressive disease versus 7.5% of disease-free patients had detectable hTERT mRNA in normal tissue [adjusted HR: 3.60 (1.64-7.94), p=0.0015]. hTERT mRNA level in tumour tissue has no prognostic value for patients with early stage lung cancer. However, detection of hTERT mRNA expression in tumour-distant normal lung tissue may indicate an increased risk of progressive disease.

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## Introduction

Lung cancer is the most important cause of cancer-related mortality worldwide (1). Non-small cell lung cancer (NSCLC) is the most common subtype, for which at present surgery is the only curative therapy. Whether a patient with NSCLC would benefit from surgical treatment is determined by staging according to the TNM system, which is currently the best predictor of outcome (2). Nevertheless, a considerable percentage of patients undergoing surgery develop metastatic disease, a local recurrence or second primary tumour. Adjuvant chemotherapy provides a survival benefit in patients that have undergone a complete (R0) resection (3-5) and is recommended for patients with post-surgical stage II-IIIA NSCLC (6). However, also ~25% of patients with stage IA disease develop a local recurrence or metastatic disease and

this subset of patients may benefit from adjuvant treatment as well (7). Molecular tumour markers may aid in determining whether a patient amenable to surgical treatment will benefit from adjuvant chemotherapy. This is illustrated by recent microarray expression and gene methylation studies suggesting that differential expression and/or methylation of certain genes in lung cancer may have prognostic value (8-11).

One candidate biomarker with potential prognostic value is increased activity of telomerase. This enzyme can extend the telomeres, which consist of 6-nucleotide repeat units that constitute the ends of the chromosomes. In the absence of telomerase activity, as is the case in most somatic cells, the telomeres shorten during every cell division and cells will lose the capacity to replicate when a critical length is reached. In most cancers, telomerase activity is increased, thereby preventing blockage of replication and allowing cells to divide indefinitely (12). The rate-limiting component of the telomerase enzyme is its catalytic subunit hTERT; the expression of which is tightly correlated with telomerase activity. Therefore, elevated hTERT mRNA levels represent a surrogate marker of increased telomerase activity in most cancers and their precursor lesions (13,14). Increased telomerase activity and hTERT mRNA levels are found in virtually all NSCLCs and are proportional to increasing severity of preneoplastic bronchial lesions (15-17). Notably, an increased hTERT mRNA level in biopsies of preneoplastic endobronchial lesions is associated with an increased relative risk of bronchial cancer or carcinoma *in situ* (18).

In patients with surgically-treated early-stage lung cancer a correlation has been found between a poor prognosis and detection in tumour tissue of either elevated hTERT expression [by RT-PCR (19-22), immunohistochemistry (23) or ISH (24,25)] or telomerase activity (26-29). However, data on this topic are not consistent since other studies failed to reveal a correlation between hTERT expression and (disease-free) survival (28,30,31). The latest study published on the subject even found elevated hTERT mRNA level to be predictive of a better prognosis (32). Whether these discrepancies reflect differences in methods or study populations is currently unknown.

The multicentre European Early Lung Cancer (EUELC) study (33) was initiated to determine whether specific genetic changes occurring in lung carcinogenesis are detectable in the respiratory epithelium of persons with an increased risk of developing lung cancer. To this end patients undergoing surgery for early-stage lung cancer were recruited in 12 cancer centres throughout Europe and followed-up at 6-month intervals. A study on FHIT gene inactivation in material from the same cohort has recently been published (34). Here, we investigated hTERT mRNA expression levels in specimens of the EUELC cohort and related these to disease-free survival.

## Materials and methods

*Study population and tissue specimens*. Between November 2002 and November 2006, NSCLC patients undergoing surgical resection for early stage (I-II) cancer were recruited in one of 12 hospitals participating in the EUELC study (Fig. 1). Inclusion criteria are detailed elsewhere (31). Of the 913 patients recruited, 210 were excluded because of insufficient follow-up (n=149), a previous history of lung- or head and neck tumour (n=29), a synchronous 2nd lung tumour (n=2) and having received (neo-) adjuvant chemo- or radiotherapy (n=30). At the time of recruitment signed informed consent was obtained and in-person structured interviews were conducted in order to gather information on demographic characteristics, smoking, medical history, occupational history (including exposure to asbestos) and family history of cancer. After surgery, patients had follow-up visits every six months for 3 years. Follow-up evaluation included chest radiography and registration of changes in smoking behaviour. Some centres also performed low-dose chest CT scans or bronchoscopy. All data were entered in an online database. The institutional review boards of participating hospitals approved this study.

In addition to formalin-fixed tumour tissue, used for routine histopathology, snap-frozen tumour tissue and, when possible, tumour-distant normal lung tissue was collected at baseline from a subset of patients. The latter involved samples of macroscopically normal lung parenchyma, taken from the surgical specimen at a maximum distance from the tumour site. All specimens were frozen within one hour following resection using either liquid nitrogen or dry ice and isopentane, and ultimately transported on dry ice to a centralized European Bronchial Tissue Bank (EBTB) in Liverpool where it was stored at -80˚C until processing.

For this study 166 patients were selected on the basis of availability of snap-frozen tumour tissue from which RNA of sufficient quality (see below) could be extracted. Mean follow-up time was 24.5±12.7 months. Characteristics of this study cohort are shown in the flowchart of Fig. 1. Though all patients revealed an apparent disease-free period of 6 months following surgery, 60 patients showed disease progression (defined as metastasis, recurrence or second primary lung cancer) during further follow-up. For most of these patients the nature of the disease post-treatment (metastasis/recurrence versus second primary cancer) was not recorded. To determine the prognostic value of hTERT mRNA parameters patients were dichotomised into two groups: progressive disease (PD) group and disease-free (DF) group, the latter comprising patients that remained tumour-free up to the last follow-up visit at 3 years. Overall mean disease-free survival was 18±18 months.

*mRNA isolation and real-time RT-PCR*. RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quality was determined by agarose gel electrophoresis and only samples with RNA of sufficient quality were used for this study. A panel of pathologists confirmed histology of tumour and normal tissue samples.

hTERT expression was assessed using the TeloTAGG GhTERT quantification method (Roche, Mannheim, Germany), a commercial real-time RT-PCR kit for use with the LightCycler (Roche, Mannheim, Germany) instrument. The house-keeping gene porphobilinogendeaminase (PBGD) was amplified in the same reaction to serve as a reference and as sample quality control. In dilution series of a telomerase-positive cervical carcinoma cell line (SiHa) hTERT mRNA

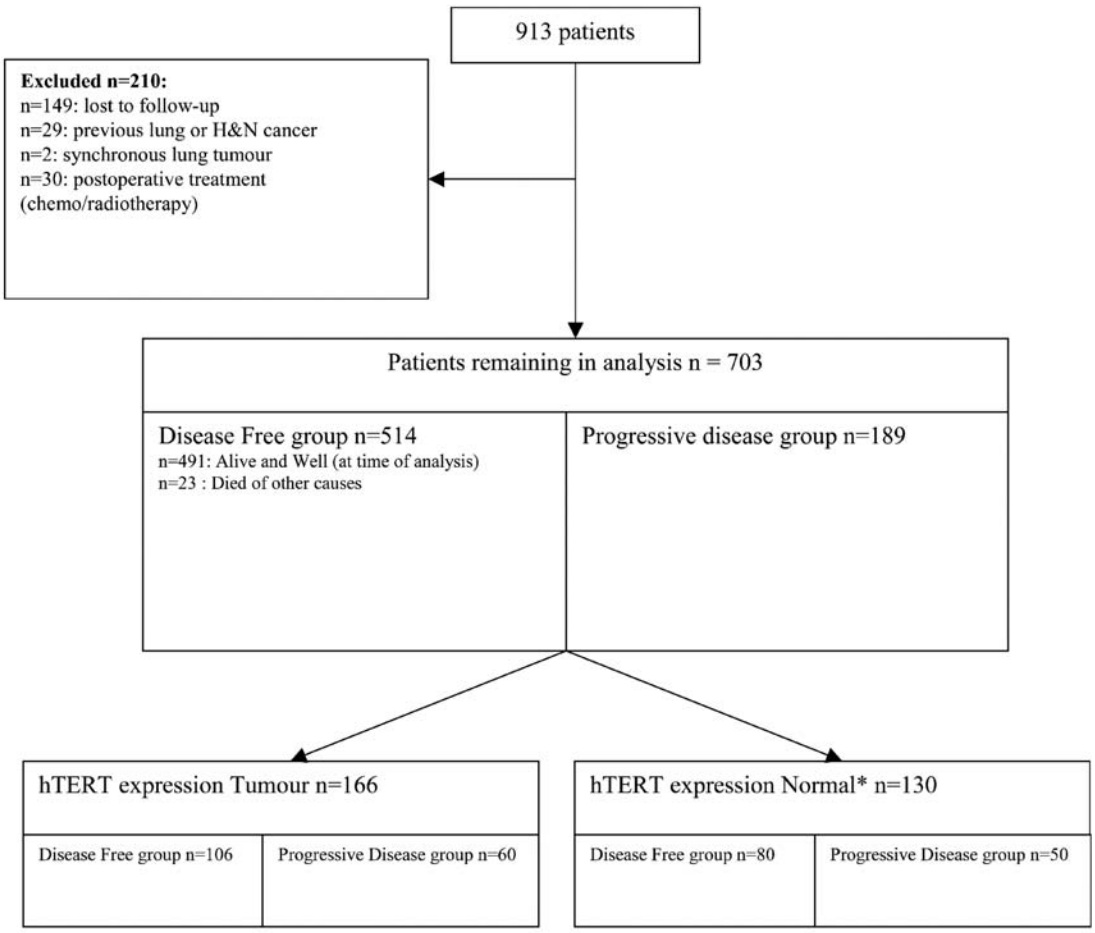


Figure 1. Flow chart. *Tumour-distant normal lung parenchyma.

was reproducibly detectable down to 3 copies hTERT mRNA per 1000 copies of PBGD mRNA, whereas at lower dilutions either no hTERT was detectable or results were highly variable, suggesting loss of reproducibility (data not shown). Consequently, for scoring samples hTERT positive versus negative a cut-off of 3 copies hTERT mRNA per 1000 copies PBGD mRNA was used.

The real-time RT-PCR was performed according to the manufacturer's recommendations. A technician who was blinded to clinicopathological and follow-up data performed the tests. The hTERT level was expressed as the number of copies per 1000 mRNA copies of PBGD.

*Statistical analyses*. Association between hTERT expression and clinical characteristics was tested using Fisher's exact test (categorical variables) or Wilcoxon non-parametric test (continuous variables). Primary end-point was disease-free survival (calculated as time from date of surgery to date of diagnosed recurrence). Univariate analysis was conducted to test the association between epidemiological information, hTERT expression and disease progression using the Fine and Gray (F&G) (35) model with stratification for centre of recruitment. The F&G model is an adaptation of Cox proportional hazard model aiming to estimate cause-specific event probabilities by censoring failures due to the competing risk event (in this case: 'Death from other causes'). Clinical variables revealed at the 5% threshold in the univariate analysis were included in a multivariate model to adjust the association with hTERT expression. All statistical analyses were performed using SAS 9.1 (SAS institute, Cary, NC).

## Results

*hTERT mRNA expression in relation to clinicopathological parameters*. RNA from frozen tumour samples of all 166 patients as well as from tumour distant normal samples available from a subgroup of 130 patients was subjected to hTERT real-time RT-PCR analysis. hTERT results in relation to clinicopathological data are shown in Table I. One hundred and twelve tumour samples (67.5%) and 20 normal samples (15.4%) tested positive for hTERT mRNA using the cut-off of 3 hTERT mRNA copies per 1000 copies of PBGD mRNA.

Amongst patients with clinical stage I and II tumours a greater proportion of hTERT positive tumour samples was stage II (Fisher's exact test p=0.016; Table I). No other variables were associated with hTERT mRNA expression in tumour or normal tissue.

The mean and median hTERT mRNA levels were higher in tumour than in normal samples, which was evident for both patients with and without disease progression (Wilcoxon non-parametric test p<0.0001, Table II). The mean hTERT mRNA levels were 42.6±78.6 and 15.8±14.3 copies per 1000

Table I. hTERT mRNA status in relation to patient characteristics.

| | Tumour samples (n=166) | | | Normal samples (n=130) | | |
|---|---|---|---|---|---|---|
| Characteristics<br>Total | hTERT$^+$<br>112 | hTERT$^-$<br>54 | p-value[a] | hTERT$^+$<br>20 | hTERT$^-$<br>110 | p-value[a] |
| Age (years) | 63.6±9.3 | 65.8±9.3 | 0.1497 | 64.3±9.1 | 63.1±10 | 0.5470 |
| Gender | | | | | | |
| Female | 24 (21.4) | 7 (13) | 0.2102 | 3 (15) | 14 (12.7) | 0.7259 |
| Male | 88 (78.6) | 47 (87) | | 17 (85) | 96 (87.3) | |
| Histology | | | | | | |
| Adenocarcinoma | 65 (58) | 30 (55.5) | 0.4443 | 12 (60) | 70 (63.6) | 0.8037 |
| SCC | 47 (42) | 23 (42.6) | | 8 (40) | 40 (36.4) | |
| Other | 0 (0) | 1 (1.9) | | 0 (0) | 0 (0) | |
| T pTNM | | | | | | |
| 1 | 35 (31.3) | 14 (25.9) | 0.2295 | 5 (25) | 45 (40.9) | 0.3717 |
| 2 | 69 (61.6) | 36 (66.7) | | 13 (65) | 61 (55.5) | |
| 3 | 6 (5.4) | 2 (3.7) | | 1 (5) | 4 (3.6) | |
| 4 | 0 (0) | 2 (3.7) | | 0 (0) | 0 (0) | |
| Unknown | 2 (1.8) | 0 | | 1 (5) | 0 | |
| N pTNM | | | | | | |
| N0 | 75 (67) | 45 (83.3) | 0.1200 | 13 (65) | 90 (81.8) | 0.2318 |
| N1 | 30 (26.8) | 8 (14.8) | | 5 (25) | 18 (16.4) | |
| N2 | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | |
| NX | 5 (4.5) | 1 (1.9) | | 1 (5) | 2 (1.8) | |
| Unknown | 2 (1.8) | 0 | | 1 (5) | 0 | |
| Tumour stage | | | | | | |
| Stage I | 72 (64.3) | 44 (81.5) | 0.0157 | 13 (65) | 88 (80) | 0.1942 |
| Stage II | 31 (27.7) | 6 (11.1) | | 4 (20) | 19 (17.3) | |
| Stage III | 2 (1.8) | 1 (1.9) | | 1 (5) | 1 (0.9) | |
| Stage IV | 0 (0) | 2 (3.7) | | 0 (0) | 0 (0) | |
| N indeterminate | 5 (4.5) | 1 (1.9) | | 1 (5) | 2 (1.8) | |
| Unknown | 2 (1.8) | 0 | | 1 (5) | 0 | |
| Pack years | 39.6±21.2 | 41.1±28.2 | 0.7302 | 40.7±23 | 41.6±24.3 | 0.9224 |
| Smoking duration | 37.8±14.6 | 38.9±13.7 | 0.6122 | 34.4±15.1 | 37.4±14.8 | 0.3387 |
| Age at initiation | 17.4±4.4 | 16.7±4.2 | 0.4804 | 15.3±2.6 | 17±4.6 | 0.0515 |
| Smoking status | | | | | | |
| Current smoker | 27 (24.1) | 10 (18.5) | 0.7639 | 7 (35) | 19 (17.3) | 0.1169 |
| Former smoker | 80 (71.4) | 41 (75.9) | | 12 (60) | 86 (78.2) | |
| Never smoker | 4 (3.6) | 2 (3.7) | | 1 (5) | 4 (3.6) | |
| Unknown | 1 (0.9) | 1 (1.9) | | 0 | 1 (0.9) | |

Data presented as mean ± SD or n (%), with percentages relative to the complete group of hTERT-positive or -negative specimens within each category; [a]Wilcoxon non-parametric test p-value for continuous variables or Fisher's exact test p-value for categorical variables (only taking into account the two largest groups, for instance AC and SCC); DF, disease-free at last follow-up; PD, progressive disease (patients with local recurrence, metastasis or second primary tumour); SCC, squamous cell carcinoma.

Table II. hTERT mRNA levels at baseline relative to disease outcome.[a]

| | Disease progression | | Disease-free | |
|---|---|---|---|---|
| | Mean ± SD | Median | Mean ± SD | Median |
| Tumour tissue (n=166) | 31.1±75.1 | 10.2 | 25.9±53.3 | 9.2 |
| Normal tissue (n=130) | 1.3±5.3 | 0 | 4.4±10.3 | 0 |
| | P<0.0001[b] | | P<0.0001[b] | |

[a]hTERT mRNA levels expressed as mRNA copy number per 1000 copies of PBGD mRNA. [b]Wilcoxon non-parametric test.

Table III. Clinical outcome as a function of hTERT mRNA expression at baseline.

| hTERT+ | n | %DF hTERT+/total (%) | %PD hTERT+/total (%) | Crude HR(95% CI) | p-value | Adjusted[a] HR(95% CI) | p-value |
|---|---|---|---|---|---|---|---|
| Tumour | 166 | 72/106 (67.9%) | 40/60 (66.7%) | 0.88 (0.50-1.56) | 0.6706 | 1.00 (0.54-1.88) | 0.9900 |
| Normal | 130 | 6/80 (7.5%) | 14/50 (28.0%) | 2.93 (1.53-5.58) | 0.0011 | 3.60 (1.64-7.94) | 0.0015 |

DF, disease-free at last follow-up; PD, progressive disease (local recurrence, metastasis or second primary tumour); HR, hazard ratio; CI, confidence interval. [a]Adjusted for tumour stage and size, smoking status and age.

Table IV. Association between hTERT mRNA expression in tumour tissue at baseline and disease progression for different hTERT cut-off values.

| Deciles (%) | Cut-off value[b] | HR (95% CI)[a] | p-value |
|---|---|---|---|
| 0 | 0 | 0.72 (0.37-1.42) | 0.34 |
| 10 | 0 | 0.72 (0.37-1.42) | 0.34 |
| 20 | 0.5 | 0.67 (0.35-1.27) | 0.21 |
| 30 | 2.9 | 0.87 (0.49-1.55) | 0.63 |
| 40 | 5.7 | 0.91 (0.53-1.57) | 0.74 |
| 50 | 10.2 | 0.81 (0.47-1.39) | 0.44 |
| 60 | 15.2 | 0.80 (0.46-1.41) | 0.44 |
| 70 | 20.4 | 0.84 (0.47-1.51) | 0.56 |
| 80 | 30.0 | 0.72 (0.36-1.45) | 0.36 |
| 90 | 60.6 | 0.83 (0.33-2.08) | 0.68 |

[a]HR, Fine & Gray hazard ratio for competing risk with centre stratification; [b]Cut-off value, number of hTERT mRNA copies per 1000 copies of PBGD mRNA.

PBGD copies for hTERT-positive tumour and normal samples, respectively.

*Correlation between hTERT mRNA expression and disease-free survival*. With the chosen cut-off of 3 hTERT mRNA copies per 1000 copies PBGD, there was no association between hTERT expression in tumour samples and disease-free survival (Table III), in a crude analysis or after adjustment for clinical variables such as stage. Similarly, when setting different hTERT mRNA cut-off values varying from the 1st to the 9th decile of hTERT levels found in tumour specimens, no association with outcome was evident (Table IV).

Instead, hTERT mRNA expression in normal lung samples was associated with an increased risk of disease progression (Table III). Of the 80 patients that remained disease-free, 6 (7.5%) had an hTERT mRNA-positive normal lung sample, versus 14 out of the 50 patients (28%) with disease progression [HR: 2.93 (1.53-5.58); p=0.0011]. This association remained significant after adjustment for clinical

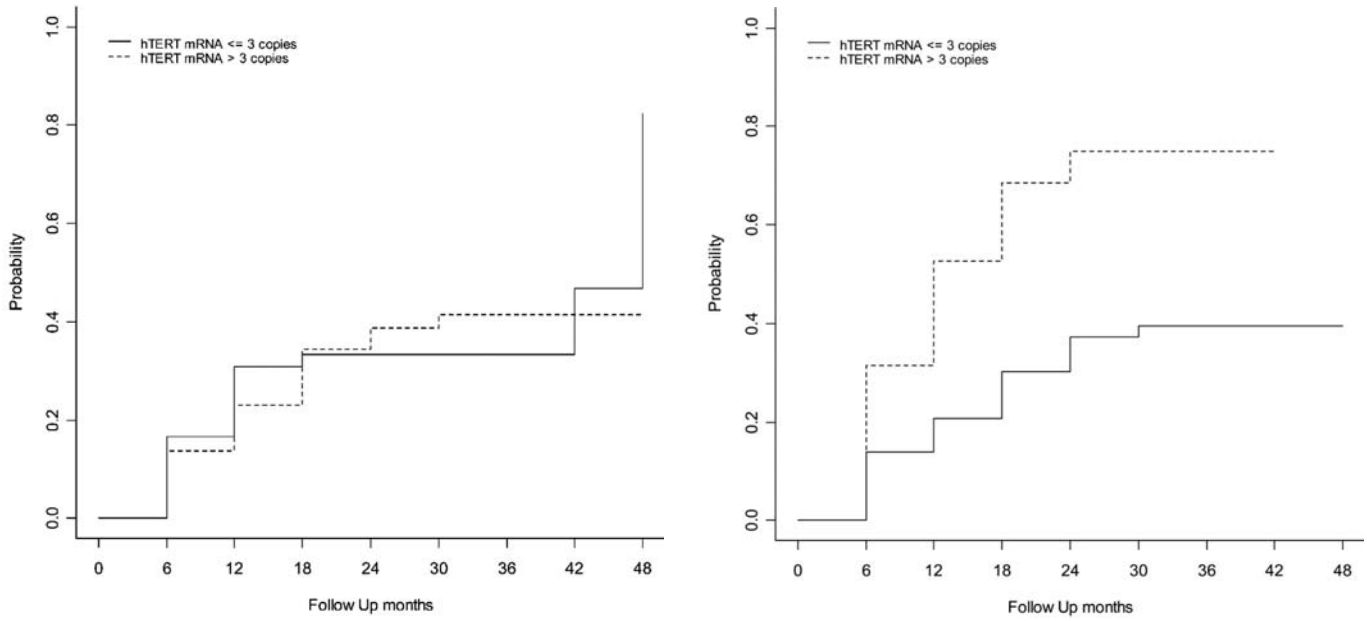


Figure 2. (A) Cumulative incidence of disease progression in relation to hTERT status of the tumour at baseline. (B) Cumulative incidence of disease progression in relation to hTERT status of tumour-distant normal tissue at baseline.

variables associated with disease progression [i.e. tumour stage and size, smoking status and age; adjusted HR: 3.60 (1.64-7.94), p=0.0015]. Cumulative incidence of disease progression in relation to hTERT mRNA status of tumour versus normal tissue is shown in Fig. 2. When normal lung samples of patients with squamous cell carcinoma and adenocarcinoma were analysed separately, an independent association between hTERT mRNA expression and progressive disease was limited to patients with SCC after adjustment (adjusted HR 5.85 CI 1.49-23.0, p=0.0120).

## Discussion

In line with previous studies we found that both the proportion of hTERT-positive samples and hTERT mRNA levels were higher in tumour samples than in paired normal lung samples of lung cancer patients (19,20,22,26-30). However, despite the fact that we examined material of a large cohort of patients and found an association between clinical stage and hTERT mRNA positivity in tumour tissue, we were unable to confirm a previously reported association between RT-PCR detected elevated hTERT mRNA levels of tumour samples and a decreased disease-free survival (19,20,22).

Telomerase activity has been reported as an independent predictor of survival of patients with varying malignancies, such as neuroblastoma (36,37), meningioma (38), breast cancer (39) and hepatic metastases of colon carcinoma (40). Studies on the potentially prognostic value of telomerase activity or hTERT expression in tumours of surgically treated lung cancer patients have, however, yielded conflicting results (19-32). The possibility that the level of hTERT mRNA and telomerase activity, rather than detectability of these parameters could have a predictive value might be one of the reasons for these discordant findings. Indeed, a correlation between increased telomerase activity (26) or elevated hTERT levels (22) in tumour samples and decreased disease-free or overall survival has been described. On the other hand, in a recent study elevated hTERT levels were found to be associated with increased disease-free survival (32). Although our study made use of a quantitative method as well, we could not confirm a relationship between tumour hTERT mRNA levels and outcome. Whether these differences are due to differences in clinicopathological characteristics of patient groups or follow-up time remains to be analysed.

Interestingly, we found that patients with detectable hTERT mRNA expression of tumour-distant normal samples had an increased risk of disease progression. Apparently, cells with a propensity to malignant outgrowth may be identified in normal lung tissue by detecting molecular changes, a phenomenon that is supported by several previous observations. Miyazu *et al* have already found that hTERT immunopositivity in epithelial cells of non-cancerous bronchial biopsies was predictive of recurrence in patients who had undergone local treatment of early lung cancer (41). In addition, gene expression profiles in cytologically normal airway epithelial cells of smokers with and without lung cancer differed in such a way that an 80-gene biomarker probe-set could be identified, displaying a high predictive value for lung cancer (42). Finally, promoter methylation of the FHIT gene in normal lung tissue, also assessed on tissue specimens of the EUELC study, was associated with an increased risk of progressive disease (34). Together, these data suggest that biomarker analysis of normal lung, specific for expression and epigenetic alterations of a panel of genes may be of value for assessment of the risk of lung cancer, and in particular of early recurrence following treatment.

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