Increased Human Telomerase Reverse Transcriptase (hTERT) mRNA Expression but not Telomerase Activity is Related to Survival in Curatively Resected Non-small Cell Lung Cancer

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Abstract. *Background: The purpose of this study was to evaluate the significance of human telomerase reverse transcriptase (hTERT) mRNA expression and telomerase activity as prognostic markers in non-small cell lung cancer (NSCLC). Patients and Methods: In a series of 69 curatively resected NSCLC specimens, telomerase activity was analyzed with the telomeric repeat amplification protocol (TRAP) assay and expression of hTERT mRNA by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Partitioning of gene expression levels and protein activities to construct prognostic groups was attempted. Results: Human hTERT mRNA transcripts were detected in 62 (89.9%) cases of NSCLC. Seven (10.1%) tumors were completely negative for hTERT expression. Dichotomized hTERT levels (<0.42 versus ≥0.42) were associated with prognosis and Kaplan-Meier survival curves demonstrated a significant difference (log rank: p<0.01) with 5-year survival rates of 44.3% (±7.1%) for low as compared to 80% (±8.9%) for high hTERT mRNA expression. Low hTERT expression was also significantly associated with squamous cell histology (p<0.03). Telomerase activity was not associated with survival, stage, pT and pN categories, histological type or grading. Comparison of hTERT mRNA expression and telomerase activity was possible in 66 patients and showed a significant difference (p<0.0001) by Wilcoxon rank test. Conclusion: This is the first study which demonstrates that high hTERT mRNA expression is associated with improved 5-year survival rates. Expression patterns are distinct among histopathological subtypes of NSCLC and telomerase activity (TRAP) is significantly higher than hTERT mRNA expression.*

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Lung cancer is one of the most common malignancies in the world and is the leading cause of cancer related deaths worldwide (1-3). Radical surgery offers the best chance for cure in patients with non-small cell lung cancer (NSCLC). Despite improvements in the detection and treatment of lung cancer in the past 2 decades, the 5-year survival rate remains <15% (3). Because pathological examination of the primary lesions cannot be used to accurately predict outcome, there is a need to identify prognostic molecular markers.

Much interest has recently focused on the enzyme telomerase and especially its subunit hTERT which has led to major advances in understanding the mechanisms of cellular proliferation, immortalization and neoplastic transformation (4).

Human telomerase consists of two major components: human telomerase RNA (hTR), which provides the template for the synthesis of the human telomeric repeat, and human telomerase reverse transcriptase (hTERT), which provides catalytic function to replicate the ends of linear DNA (5). The presence of both subunits is mandatory for telomerase activity. Although hTR is constitutively expressed in all tissues, hTERT expression seems to be restricted to telomerase-positive tissues, indicating that hTERT is the limiting factor for telomerase activity (6). Telomere

stabilization can also be achieved by mechanisms that are independent of telomerase. However, these mechanisms referred to as alternative lenghtening of telomeres have not been elucidated yet (7).

Reactivation of telomerase activity in most types of human cancer provides the potential of measuring telomerase activity or its subunits as a general molecular marker for malignant transformation and immortalization. Following the introduction of a PCR-based assay, called the telomeric repeat amplification protocol (TRAP), telomerase activity was found to be present in most human tumors (8). Since the cloning of the main constituent of the telomerase complex, hTERT, research had an additional tool for analyzing the role of telomerase in human cancer (9). Meanwhile there are reports about various tumors, including urothelial cell carcinoma (10), colorectal carcinoma (11) and breast cancer (12), which claim that assessment of *hTERT* mRNA expression levels may be used as a negative prognostic marker. However, the prognostic significance of *hTERT* expression or activity in NSCLC remains controversial: while most studies have associated overexpression with poor prognosis (13-17), others failed to demonstrate any prognostic impact of this factor in NSCLC (18-22). Therefore, the purpose of this study was to evaluate the significance of both human telomerase reverse transcriptase (hTERT) expression and telomerase activity as prognostic markers in potentially curative (R0) resected NSCLC.

Patients and Methods

Study population. Specimens were used of paired tumor and normal lung tissues from 69 patients with NSCLC that were available from a previous prospective study of 103 consecutive patients with completely resected (R0-Resection) NSCLC in histopathological stages I-IIIA according to the Union Internationale Contre Le Cancer (UICC) Tumor Node Metastasis (TNM) Classification (23, 24).

There were 52 (76%) men and 17 (24%) women with a median age of 63.3 years (range: 34-82 years). Thirty-four (49%) patients had squamous cell carcinomas (SCC), 27 (39%) adenocarcinomas (AC), and 8 (12%) had large cell carcinomas (LCC). The primary tumors were graded histopathologically as well differentiated (G1, n=1), moderately differentiated (G2, n=15), and poorly differentiated (G3, n=53). Tumor staging was performed according to the UICC TNM Classification (17): 37 (54%) had stage I tumors, 12 (17%) stage II and 20 (29%) had stage IIIA tumors. All of the tumors were radically removed by lobectomy (n=45), bilobectomy (n=9), pneumonectomy (n=11) and extended pneumonectomy (n=4), including mediastinal lymphadenectomy for all of the procedures. Patients with histopathological stage IIIA tumors received postoperative radiotherapy. The median follow-up was 7.2 years (range: 0.3-8.8 years). Patients were seen at 3-month intervals during the first postoperative year, every 6 months in the second and third year and once a year thereafter. Evaluation consisted of physical examination, biochemical profile, chest radiograph, computed tomography (CT) scan of brain, chest and abdomen, abdominal ultrasound and technetium bone scan. No patient was lost to follow-up. Data on recurrences and cause of death were obtained for all patients and informed consent was obtained from all of the patients.

Tissue acquisition. Tissue samples for DNA and RNA analysis were obtained immediately after lung resection before starting mediastinal lymphadenectomy and were frozen in liquid nitrogen and stored at –80˚C until further processing. Tissue was analyzed from the following 2 locations: tumor and uninvolved lung tissue taken from the greatest distance to the tumor. Tumor tissue was selected from areas with at least 75% malignant cells.

RNA isolation and cDNA synthesis. Total cellular RNA was extracted from frozen specimens using Trizol reagent (Life Technologies, Gibco BRL, Grand Island, NY, USA). The obtained RNA was quantified spectrophotometrically at $A_{260/280\ nm}$ (Smart Spec, Biorad, Hercules, CA, USA). A total of 1 μg RNA from each sample was reverse-transcribed using oligo $d(T)_{18}$ primer and MMLV (Moloney murine leukemia virus) reverse transcriptase (Clontech, Palo Alto, CA, USA) according to the protocol of the manufacturer. The cDNA was diluted to a total volume of 200 μl.

Real-time RT-PCR. Human *TERT* mRNA expression in the tumors and corresponding normal tissue was measured by real-time quantitative RT-PCR, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). By means of fluorescence emission, this technique allows the threshold where a specific PCR product becomes detectable to be identified. This Ct value correlates to the starting quantity of the target (25, 26). To normalize the amount of total RNA present in each reaction, the housekeeping gene *GAPDH* was amplified.

Design of primers and probes. Sequence information was obtained from Genebank and previously published data (9). Primer and probes were designed using a standardized procedure: First, cDNA sequences for full length clones were blasted against human genomic sequences to identify exon-exon junctions. Primer and probes were designed using the Primer Express software (Applied Biosystems). The resulting primer and probe sequences were verified in BLAST (NCBI). The GAPDH probe was labelled with 5'-VIC and 5'-TAMRA. GAPDH primers and probe have been described elsewere (27). The *hTERT* probe was labelled at the 5' end with FAM and at the 3' end with the quencher TAMRA (Eurogentec, Seraing, Belgium). The sequences for the hTERT primer and probe were: forward 5'-TGTGCACCAACATCTACAAGATCC, reverse: CTGATGAAATGGGAGCTGACG, probe: TGCTGCAGGCGTAC AGGTTTCACG. Each Probe was analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and reliability of PCR amplification and detection was verified on serial dilutions of standard cDNAs prior to analysis of patient samples. When genomic DNA was used as a template, no bands were detected after PCR amplification.

Analyses of gene expression by real-time quantitative RT-PCR. Expression levels of *hTERT* and the housekeeping gene *GAPDH* were quantified using a fluorescence-based real-time detection method (ABI PRISM 7900). The PCR reaction mixture contained 600 nM of each primer, 200 nM probe in a final volume of 22.5 μl. PCR conditions were 50˚C for 10 s, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min. The initial template

was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction (Ct-value). Relative gene expression levels were calculated using standard curves generated by serial dilutions of a mixture of cDNAs representing a wide variety of normal and tumor samples. The relative expression amounts were calculated by using the expression of *GAPDH* as an internal standard. All genes were independently analyzed twice and reproducibility was excellent with a correlation coefficient r>0.95.

TRAP assay. For the detection of telomerase activity, a commercially available photometric enzyme immunoassay using the telomeric repeat amplification protocol as supplied was used (Telo TAGGG Telomerase PCR ELISA; Roche Applied Science, IN, USA). The test was performed according to the manufacturer's protocol. Briefly, 5 µg total protein extracted from tissue specimens was used for each combined primer elongation/amplification reaction. Five microliters of the amplification product was transferred for the hybridization and ELISA procedure. The ELISA was analyzed within 30 minutes after the addition of stop reagent using an ELISA Reader at 450 nm and 690 nm reference wavelength (Microplate Reader Benchmark; Bio-Rad Laboratories, Munich, Germany). Each assay contained a positive and negative control (protein extract of a melanoma cell line). Samples were regarded as telomerase-positive if the difference in absorbance between sample and negative control was higher than 0.2. All measurements were performed in duplicate.

Statistical analysis. Gene expression levels (mRNA) and telomerase activities were described using the median as point estimator and the range of values.

For the identification of the best cut-off value for a prognostic variable the log-rank test was simulated for all observed values within the entire data set according to LeBlanc and colleagues (28). Associations between dichotomized mRNA and protein expression levels and clinicopathological parameters were evaluated using χ^2-analysis and Fisher's exact test for significance.

Kaplan-Meier plots were used to describe the survival distributions and the log-rank test was used to identify significant survival differences (29). Cox regression analysis was the method applied when several factors were assessed simultaneously (30). The level of significance was set to $p<0.05$.

All statistical tests were performed using the Software Package SPSS for Windows, Version 11.0, Chicago, IL, USA.

Results

Clinical data. In this study, NSCLC specimens from 69 patients were analyzed. Median and 5-year survival rates depending on various clinical variables are summarized in Table I. Histopathological UICC tumor stage ($p<0.0001$), pT category ($p<0.04$) and pN category ($p<0.00001$) were of significant prognostic importance. Gender, age, histologic subtype and grading of the primary tumor had no prognostic impact on survival.

Human TERT mRNA expression analysis. Human *TERT* mRNA transcripts were detected by quantitative RT-PCR in 62/69 (89.9%) NSCLC. In 7/69 (10.1%) tumors, *hTERT* mRNA could not be detected. In none out of 14 randomly picked paired normal lung specimens (control group) was *hTERT* mRNA detected. Median *hTERT* expression in the tumor samples was 0.17 (range: 0.00-9.51). Optimal cut-off values were calculated for *hTERT* mRNA expression at <0.42 (low) *versus* ≥0.42 (high) for discrimination of survival probabilities according to LeBlanc *et al.* (23). Kaplan-Meier survival curves are shown in Figure 1 and demonstrate a significant difference ($p<0.01$) with 5-year survival rates of 44.3% (±7.1%) for low compared to 80% (±8.9%) for high *TERT* mRNA expression.

Dichotomized *hTERT* mRNA levels (<0.42 *versus* 0.42) were not significantly associated with histopathological stage ($p=0.28$), pT ($p=0.63$), pN categories ($p=0.41$) or grading of the tumor ($p=0.35$). In contrast, there was a significant association of *hTERT* mRNA expression with the histological subtype ($p<0.03$). The data shown in Table II demonstrate that low *hTERT* expression (<0.42) was more frequently observed in squamous cell cancer (85.3%) compared to adenocarcinoma (59.3%) or large cell carcinomas (50%).

TRAP assay. Telomerase activity was measured in 66 paired tumor and normal lung tissues. The median telomerase activity was 0.99 (range: 0-2.18) in tumors and 0.1 (range: 0-1.29) in paired normal lung tissue. Telomerase activity was significantly lower in normal tissues as compared to tumors ($p<0.0001$).

Table I. *Survival in NSCLC based on clinical parameters.*

Parameter	n	5-Year survival (%) ±S.D.	Median survival (months)	95% CI	*p*-Value (log rank)
UICC Stage					
I	37	70.4±8.2	n.r.	-	
II	12	56.5±14.6	n.r.	-	0.0001
III A	20	15.0 ±7.9	18.8	13.8-23.9	
pT					
pT_1	13	76.9±11.6	n.r.	-	
pT_2	45	50.6±8.1	n.r.	-	0.04
pT_3	11	27.2±13.4	26.7	12.3.23.4	
pN					
pN_0	42	69.2±7.7	n.r.	-	
pN_1	16	41.9±12.5	33.9	12.7-55.3	0.00001
pN_2	11	0	16.7	9.9-23.4	
Histology					
SCC	34	61.1±8.4	n.r.	-	
AC	27	44.4±9.5	51.3	27.1-75.6	0.48 (n.s.)
LC	8	41.6±20.4	63.9	39.5; 94.3	
Grading					
G_1	1	(n=1)	-	-	
G_2	15	46.7±12.8	59.7	43.9-79.8	0.28 (n.s.)
G_3	53	54.3±7.3	68.2	56.5-79.9	

n.r., Not reached; -, cannot be calculated; 95% CI, 95% confidence interval; n.s., not significant; n, number of patients; SCC, squamous cell cancer; AC, adenocarcinoma; LC, large cell cancer.

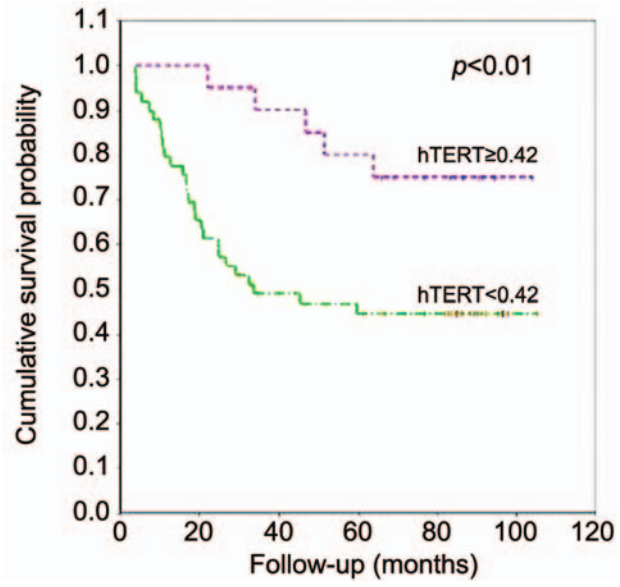

Figure 1. *Kaplan-Meier curves including NSCLC specimens from 69 patients based on dichotomized optimal cut-off values for hTERT mTNA expression. A total of 49 tumors showed low (<0.42) and 20 high (≥0.42) hTERT mRNA expression levels. Human TERT mRNA expression ≥0.42 is significantly associated with improved survival rates (log-rank: p<0.01).*

Telomerase activity was not associated with survival and no cut-off value for prognostic partitioning could be detected. There was also no significant association of telomerase activity in tumors with UICC stage, pT and pN categories, histological type or grading.

Association of hTERT expression and telomerase activity in NSCLC. Comparison of *hTERT* mRNA expression and telomerase activity (TRAP assay) was possible in 66 patients and showed a significant difference (Wilcoxon rank test: $p<0.0001$). This was due to 49 ranks with TRAP activity >*hTERT* mRNA expression compared to 18 with TRAP activity <*hTERT* mRNA expression and one binding.

Discussion

Over the past few years, numerous studies have shown that telomerase is expressed and active in various types of tumors including NSCLC (10-13). Although several studies reported telomerase to be an independent marker of poor prognosis in different tumor entities, contradictory results have also been shown (13-22).

The aim of this study was to further elucidate the association between clinicopathological parameters, telomerase activity and *hTERT* mRNA levels in patients with curatively (R0 resection according to UICC criteria) resected NSCLC using the TRAP and a quantitative real-time RT-PCR assay for *hTERT* analysis.

Table II. *hTERT mRNA expression and histological type.*

	Histologic subtype		
hTERT[a]	SCC	AC	LC
<0.42: n (%)	29 (85.3%)	16 (59.3%)	4 (50%)
hTERT ≥0.42: n (%)	5 (14.7 %)	11 (40.7%)	4 (50%)
Total: n (%)	34 (100 %)	27 (100%)	8 (100%)

SCC, squamous cell cancer; AC, adenocarcinoma; LC, large cell cancer. [a]Dichotomized hTERT mRNA levels; χ^2-analysis (Fisher's exact test): $p=0.03$.

We detected *hTERT* mRNA in 89.9% of NSCLC specimens and in none of 14 randomly analyzed paired normal lung specimens. This observation is in agreement with previously published reports (18, 21).

For partitioning of prognostic groups based on dichotomized *hTERT* mRNA expression, an optimal cut-off value of 0.42 was detected with significant prognostic impact. To the best of our knowledge, this is the first study which demonstrates a survival benefit for patients with tumors with high hTERT mRNA expression. A comparison of results between various reports is difficult, mainly because of the different techniques used for the detection of *hTERT*. Conventional gel based RT-PCR, real-time RT-PCR, *in situ* hybridisation (ISH) and immunohistochemistry (IHC) techniques have been applied. There is one report from Marchetti *et al.* (14) which also applied real-time quantitative RT-PCR technology. Contrary to our results, they found that in stage I NSCLC patients, *hTERT* mRNA expression levels were significantly associated with reduced survival probability. Zhu *et al.* (17) was only able to show that *hTERT* mRNA overexpression was associated with reduced recurrence-free survival. On the other hand, Lu *et al.* did not show a prognostic significance of hTERT mRNA expression in resected stage I non-small-cell lung cancer patients when performing a multivariate analysis of six molecular markers (22). In addition, two studies (18, 21) using conventional gel-based RT-PCR could not find any association with clinicopathological parameters including survival probabilities.

In situ hybridization was also used for the detection of hTERT and Wang *et al.* (13) demonstrated a survival benefit for patients with stage I disease and low *hTERT* expression. For stage I patients, this result was confirmed by Fujita and coworkers (15), whereas in patients with stage II and III disease no significant difference between the groups with strong and low *hTERT* staining could be detected. However, immunohistochemical analyses by Kumaki *et al.* for the detection of telomerase protein expression did not identify any statistical evidence for an association of survival probabilities and levels of hTERT protein expression in

NSCLC (19). In the study of Wang and colleagues (13), one major concern is the very low percentage (33%) of hTERT-positive NSCLC in contrast to all other cited studies which report 93.9% (19) up to 100% (15) of positive cases.

At this point, we can only speculate as to the reasons for these discrepant findings. Among possible reasons are a) differences of TRAP and hTERT assays performed in previous studies; b) lack of statistical power due to limited number of telomerase-negative patients and the total number of study patients.

With respect to histology, we demonstrated a significant association of *hTERT* mRNA expression with the histological subtype ($p<0.03$). Our data show that low *hTERT* expression (<0.42) is more frequently observed in squamous cell cancer (85.3%) compared to large cell (50%) or adenocarcinomas (59.3%). Lantuejoul *et al*. (16) analyzed telomerase expression with respect to NSCLC histology. They utilized polyclonal and monoclonal antibodies raised against hTERT protein and found significantly lower telomerase expressions in AC than in SCC.

Furthermore, we found a significantly higher telomerase activity compared to *hTERT* mRNA expression levels applying the Wilcoxon rank test in 66 paired samples. Marchetti *et al*. (14) also reported a statistical significant link between *hTERT* gene expression and telomerase activity ($p=0.017$). In his study, patients with both *hTERT* gene expression and missing telomerase activity (21/90) had a statistical significant better disease-free ($p=0.0092$) and overall survival ($p=0.0095$).

In summary this is the first report about the association of high *hTERT* mRNA expression and a favourable prognosis in completely resected NSCLC and this contradicts some previous reports. Therefore, large trials are needed to improve our knowledge about telomerase regulation in order to clarify contradictory results concerning the prognostic impact of *hTERT* RNA expression and telomerase activity in NSCLC.

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