ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 4/2017
641–646
https://doi.org/10.18388/abp.2017_1618

Regular paper

Clinical and prognostic value of *hTERT* mRNA expression in patients with non-small-cell lung cancer

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Telomerase, undetectable in normal somatic cells, plays a critical role in carcinogenesis of the majority of human tumors including lung carcinoma. The aim of our study was to determine human telomerase reverse transcriptase (*hTERT*) mRNA expression in patients with non-small cell lung cancer (NSCLC) in order to estimate its usefulness as diagnostic and/or prognostic factor. *hTERT* expression was analyzed in a group of 12 females and 28 males with NSCLC using Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR method) in cancerous and non-cancerous lung tissues. Results were analyzed according to clinical data and one-, two-, and five-year survival rates. *hTERT* expression in the cancerous tissue was significantly higher than in the lung parenchyma free from neoplasm infiltration ($p<0.05$). There was no significant association between *hTERT* expression in the tumor tissue and age, gender, grading or clinical stage. A significant difference in *hTERT* expression between two types of histopathological patterns (adenocarcinoma and squamous cell carcinoma) was detected ($p=0.01$). No association between *hTERT* expression in NSCLC specimens and survival rates was found. *hTERT* mRNA detection by QRT-PCR in tumor and corresponding cancer-free tissues can be used as a diagnostic marker in patients with NSCLC, but seems not to be a prognostic factor.

Key words: non-small-cell lung cancer, telomerase, hTERT expression, survival rate, prognosis

Received: 03 June, 2017; revised: 27 July, 2017; accepted: 10 September, 2017; available on-line: 15 November, 2017

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Abbreviations: hTERT, human telomerase reverse transcriptase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NSCLC, non-small-cell lung cancer; SCC, squamous cell carcinoma; pTNM, pathological tumor-node-metastasis staging; G, grading; cDNA, complementary deoxyribonucleic acid; RNA, ribonucleic acid; RT, reverse transcription; QRT-PCR, quantitative real time-polymerase chain reaction

INTRODUCTION

Lung cancer is one of the most important cause of cancer-related mortality worldwide, and the most frequent cause of deaths in Poland (Parkin *et al*., 2005; Radziszewska *et al*., 2015). The WHO has categorized lung cancers into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) based on cancer biology, therapy responsiveness and prognosis (Goldstraw *et al*., 2007). NSCLC, diagnosed as three common types: adenocarcinoma, squamous cell carcinoma and large cell carcinoma, accounts for more than 85% of all lung cancer cases (Ettinger *et al*., 2010). For these lung cancer types surgery is the only therapeutic option nowadays. Due to the low effectiveness of the therapy and high mortality of patients with NSCLC, there is still a need to discover markers useful either in diagnostics and qualification for surgical treatment or in prognostics.

Telomeres are DNA–protein structures located at the 3' ends of DNA strands, composed of repeated guanine-rich sequences 5'-TTAGGG-3' and stabilized by an associated protein (shelterin complex). Their role is to protect chromosome ends from degradation by exonucleases and prevent non-homologous end joining; therefore, they are essential for the maintenance of genomic integrity (Armanios *et al*., 2012; Mocellin *et al*., 2013; Palm *et al*., 2008). The telomeres' length is set in the germline but decreases in somatic cells, primarily as a result of the inability of DNA polymerase to complete replication of the lagging strand ("end replication problem"). Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by catalyzing the addition of TTAGGG repeats. Human telomerase consists of two major components (protein and RNA): human telomerase reverse transcriptase catalytic subunit (hTERT) and a functional telomerase RNA (hTR, hTERC) (Palm *et al*., 2008; Shay *et al*., 2000; Shay *et al*., 2005; Weinrich *et al*., 1997). Telomerase uses its RNA component (which contains an 11-bp sequence complementary to the telomeric single stranded overhang) as a template for synthesizing telomeric cDNA $(TTAGGG)_n$ directly onto the ends of chromosomes. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells, resulting in progressive shortening of telomeres. Telomere shortening to a critical length results in the loss of telomere protection, which leads to chromosome instability, stops cell division and replicative senescence may be initiated (Shay *et al*., 2005). Some cells, however, exhibit unlimited capacity for proliferation through the telomerase complex activity (Palm *et al*., 2008; Weinrich *et al*., 1997). In the large majority of cancer cells, telomere length is maintained by telomerase. Thus, telomere length and telomerase activity are crucial for cancer initiation and the survival of tumors (Jafri *et al*., 2016). Several studies on the relationship between telomerase activity and prognosis in lung cancer have been reported (Hara *et al*., 2001; Marchetti *et al*., 1999). The expression of *hTERT* was frequently detected in lung cancer as was telomerase activity (Albanell *et al*, 1997; van den Berg *et al*., 2010; Wang *et al*., 2002) but the results of these studies are ambiguous.

The aim of this study was to determine the clinical significance of human telomerase reverse transcriptase (*hTERT*) mRNA expression in patients with NSCLC and to evaluate its usefulness as a diagnostic parameter and a prognostic factor in this group of patients.

MATERIALS AND METHODS

Patients and samples. A group of 40 patients diagnosed with NSCLC and treated in the Thoracic Surgery Ward of the Specialist Hospital of Lung Diseases and Tuberculosis in Bystra Slaska in 2009–2010 were enrolled into the study. The cohort of patients included 12 females (30%) and 28 males (70%), aged 47 to 75 (mean: 63.5±8.0). Clinical and pathological features of the patients are presented in Table 1.

During surgery, specimens of the tumor tissue and the lung parenchyma free from neoplastic infiltration (taken no less than 5 cm from the visible edge of the tumor) were obtained. Each sample was immediately snap-frozen in a separate tube with liquid nitrogen and stored at -80°C until it was used for quantitative real-time PCR (QRT-PCR). After that the samples were transported on dry ice for further examination at the Molecular Research Laboratory of the Department of Medical and Molecular Biology in Zabrze.

The advancement of NSCLC was determined with the clinical TNM scale and histopathological examination of tumor cell differentiation grading (G) made independently by two pathomorphologists.

RNA extraction, reverse transcription and QRT-PCR for hTERT and a housekeeping gene GAPDH. Isolation of the total RNA from about 80 mg of frozen tissue samples was performed with the RNeasy® Midi Kit (Qiagen, Germany). Apart from the standard procedure, DNase I (Qiagen, Germany) was used to remove trace amounts of genomic DNA. RNA was quantified by measuring the absorbance at 260 and 280 nm and the integrity was assessed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. RNA isolates were used to synthesize cDNA in the RT reaction. 200 ng of total RNA was transcribed into cDNA in a total volume of 20 µl by the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) following the manufacturer`s instruction. Obtained cDNA was used to determine *hTERT* gene expression using a quantitative real-time PCR assay (TaqMan® system). TaqMan® primers and a probe for *hTERT* were bought as ready to use TaqMan® Gene Expression Assays (Hs00972656_m1) and for the housekeeping gene GAPDH Endogenous Control (Hs02758991_g1) (Applied Biosystems, USA). Q-PCR was conducted for both genes in a volume of 20 µl on the ABI PRISM 7300 Real Time PCR Detection System (Applied Biosystems, USA). Each time, a Q-PCR mix was prepared on ice with 10 µl of Applied Biosystems Universal PCR Master Mix, 1 µl of "primers and probe mix", and 8 µl of H_2O (Qiagen, Germany). To each well of a 96-well plate, 19 µl of Q-PCR mix and 1 µl of cDNA were added. All PCRs were conducted in triplicates. A negative control was included for all amplification reactions. *hTERT* and *GAPDH* genes were amplified starting with the incubation step at 50°C for 2 minutes, followed by the first denaturation step at 95°C for 10 minutes, and continuing with 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Gene expression was considered negligible when the Ct (threshold cycle) value was greater than 40. In view of the heterogeneity of the investigated tissue and the lack of ability to use a commercially available standard, to determine *hTERT* expression the ΔC_T method was applied (Livak *et al*., 2001).

Thanks to the kindness of the Document Personalization Centre of the Ministry of Internal Affairs and Administration in Warsaw, Poland, we got access to information about the patients' dates of death, which were needed to calculate survival time. Thus, 1-, 2-, and 5-year survival rates were calculated and then correlated with *hTERT* expression in cancerous and non-cancerous tissues.

Statistical analyses. Statistical analyses were performed to examine the correlation between *hTERT* mRNA expression and the clinical parameters of NSCLC patients. Qualitative variables are presented in absolute terms (number of patients showing the presence of a variable) and as a percentage of the entire analyzed study group, while qualitative variables were expressed as a mean and a standard deviation (S.D.). The obtained data were statistically analyzed based on the Student's *t*-test or Mann-Whitney U-test ($p<0.05$ was considered statistically significant). Pearson, Kendall and Spearman rank correlation coefficients were used to detect monotonic and non-monotonic correlations in this study. Survival curves were calculated by the Kaplan-Meier method and were compared using Gehan's generalized Wilcoxon test. Statistical analyses were performed using the STATISTICA 12.0 software (StatSoft. Inc., Tulsa, OK, USA). The study protocol was approved by the Ethical Committee of the Medical University of Silesia in Katowice, Poland (KNW/0022/

Table 1. The clinical and pathological features of 40 NSCLC patients.

Variable	Number of patients (%)
Total number	40 (100)
Age	
≤65	21 (52.5)
>65	19 (47.5)
Histology	
Squamous cell carcinoma	29 (72.5)
Adenocarcinoma	11 (27.5)
Large cell carcinoma	0 (0.0)
Differentiation grade	
G1	0 (0.0)
G2	17 (42.5)
G3	23 (57.5)
T factor	
T1	9 (22.5)
T2	24 (60.0)
T3	6 (15.0)
T4	1 (2.5)
N factor	
N0	20 (50.0)
N1	14 (35.0)
N2	6 (15.0)
Clinical stage	
I	13 (32.5)
II	17 (42.5)
III	8 (20.0)
Undefined	2 (5.0)
Survival rates	
one-year	28 (70.0)
two-year	26 (65.0)
five-year	25 (62.5)

The abbreviations: G, histopathological cancer grading (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated); T, tumor size; N, metastatic lymph nodes.

KB1/119/I/09). Informed consents were obtained for all study participants.

RESULTS

The study group characteristics are shown in Table 1. The expression of *hTERT* was detected in 39 out of 40 (97.5%) of surgery excised NSCLC tissue while in visually normal lung tissue it was observed in 24 out of 32 specimens (75%). Moreover, the average *hTERT* expression (average ΔC_T) in neoplastic tissue was 0.000665, and it was significantly higher than the average *hTERT* expression in the parenchyma of the lung without tumor infiltration (0.000189), ($p=5.24\times10^{-4}$). A significant difference in *hTERT* expression between two types of histopathological patterns (adenocarcinoma and SCC) was also detected (p=0.01). On the other hand, no significant differences in *hTERT* expression in tumor tissues between gender, age, grading (G), clinical stage, size of the tumor (T) and degree of involvement of regional lymph nodes (N) were observed (Table 2). Significant differences in *hTERT* expression in non-cancerous tissues between gender, age, histological type, grading, clinical stage, T and N parameters were not observed (Table 3). A correlation between *hTERT* expression in the tumor and *hTERT* in non-cancerous tissues was not found.

During the five-year observation period, one-year, two-year, and five-year survival rates of the 40 patients treated with surgery were evaluated. Within one year after surgery 12 patients (30%), within two years 14 patients (35%), and within five years 15 patients (37.5%) died due to the progression of the disease. Individual survival rates are presented in Table 1. Comparing *hTERT* expression in tumor tissues of still living and deceased patients we did not find important differences one year, two, and five years after surgery (Table 2).

Subsequently we analyzed the relationship between *hTERT* expression in non-cancerous tissues and length of survival. There were no significant differences in

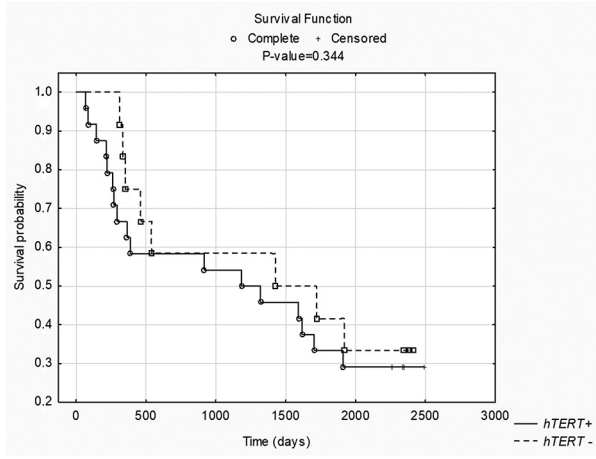

Figure 1. Survival analysis of patients with NSCLC based on the classification of *hTERT*-positive and -negative mRNA expression in non-cancerous tissues.
The figure shows overall survival curves analyzed using the Kaplan-Meier method.

Table 2. *hTERT* mRNA expression in NSCLC tumor specimens depending on various clinical and pathological factors.

Clinical and pathological factors		N	%	Average hTERT expression (ΔC_t)	*p*
Gender	F	12	30.00	0.000692	0.88
	M	28	70.00	0.000654	
Age	≤65	21	52.50	0.000857	0.08
	>65	19	47.50	0.000454	
Type of histological pattern	squamous cell carcinoma	29	72.50	0.000494	0.01
	adenocarcinoma	11	27.50	0.001116	
Grading	G2	17	42.50	0.000653	0.93
	G3	23	57.50	0.000674	
Clinical stage	I	13	32.50	0.000473	0.30
	II–III	25	62.50	0.000729	
T: Primary tumor	T1	9	22.50	0.00036	0.14
	T2–T3	30	75.00	0.00077	
N: Lymph nodes	N0	20	50.00	0.000613	0.66
	N1-N2	20	50.00	0.000717	
One-year survival	yes	28	70.00	0.000683	0.82
	no	12	30.00	0.000624	
Two-year survival	yes	26	65.00	0.000642	0.78
	no	14	35.00	0.000709	
Five-year survival	yes	25	62.50	0.000644	0.81
	no	15	37.50	0.000701	

Table 3. *hTERT* mRNA expression in non-cancerous lung specimens depending on various clinical and pathological factors.

Clinical and pathological factors		N	%	Average *hTERT* expression (ΔC_t)	*p*
Gender	F	9	28.12	0.000174	0.73
	M	23	71.88	0.000195	
Age	≤65	15	46.88	0.000189	1.00
	>65	17	53.12	0.000189	
Type of histological pattern	squamous cell carcinoma	22	68.75	0.00017	0.29
	adenocarcinoma	10	31.25	0.000232	
Grading	G2	11	34.38	0.000162	0.52
	G3	20	62.50	0.0002	
Clinical stage	I	8	25.00	0.000222	0.45
	II–III	23	71.88	0.000174	
T: Primary tumor	T1	6	19.35	0.000232	0.47
	T2–T3	25	80.65	0.00018	
N: Lymph nodes	N0	13	40.62	0.000226	0.26
	N1–N2	19	59.38	0.000164	
One-year survival	yes	22	68.75	0.000177	0.50
	no	10	31.25	0.000217	
Two-year survival	yes	17	53.13	0.000148	0.10
	no	15	46.87	0.000236	
Five-year survival	yes	16	50.00	0.000154	0.19
	no	16	50.00	0.000225	

hTERT expression between living and deceased patients one, two, and five years after surgical treatment (Table 3). The overall survival curves analyzed using the Kaplan-Meier method are shown in Fig. 1. During the 5-year observation period, the probability of survival between the group of patients showing *hTERT* expression in non-cancerous tissues and the group not exhibiting such expression was not significantly different (p=0.33).

DISCUSSION

Currently the best predictor of outcome is the pTNM system and it has been shown that benefits from surgical treatment are determined by lower staging at the moment of diagnosis (van den Berg *et al.*, 2010). Unfortunately, the overwhelming part of Polish patients are diagnosed at advanced stages of lung cancer. Thus, they cannot be offered a radical surgical treatment. Recent data show that only about 16% of patients are qualified to surgical procedures. Consequently, the five-year survival rates in our country are highly unsatisfactory (Radziszewska *et al.*, 2015).

Among more than 100 proposed cancer markers, telomerase is detected in almost all kinds of cancer tissues. Telomerase activity has been found in approximately 85–90% of the most common cancers such as breast, prostate, lung, liver, pancreatic, and colon cancers (Hiyama *et al.*, 2001; Kim 1997; Shay *et al.*, 1997). As telomerase activity appears in most tumor cells at the preneoplastic or *in situ* stage, it may be useful for early detection of cancer, especially in cytology samples. In other instances, in which the level of telomerase activity is not high but increases with cancer progression, telomerase activity levels in tumor tissues may be prognostic indicators of the patient's outcome (Hiyama *et al.*, 2002; Shay *et al.*, 1997).

In our previous study (Dobija-Kubica *et al.*, 2016) we showed even though the telomerase activity was significantly higher in NSCLC tissue specimen than in the lung parenchyma free from tumor infiltration, there was a lack of significant differences according to other clinical parameters. This suggests that telomerase activity is connected only with the tumor cell proliferation. On the other hand, the variation in telomerase activity in cancer tissues might be associated with different levels of telomerase expression in each tumor cell. The difference may result from the absolute number of tumor cells that show telomerase activity (Hiyama *et al.*, 2001). Therefore, the present study assessed the utility of *hTERT* expression as a diagnostic and/or prognostic factor.

The presence of *hTERT* mRNA is essential for enzymatic activity so the expression of *hTERT* correlates with telomerase activity (Hara *et al.*, 2001; Kumaki *et al.*, 2001). Thus, detection of the *hTERT* mRNA is considered a more reliable marker of the presence of cancer cells in clinical samples (Chen *et al.*, 2011). In our study *hTERT* expression was detected in 97.5% (39/40) of

NSCLC tumor specimens and in 75% (24/32) of non-cancerous specimens and it was significantly higher in cancerous tissues ($p=5.24\times10^{-4}$). Our results are consistent with other authors (Hara *et al*., 2001; van den Berg *et al*., 2010). Fujita *et al*. suggested that *hTERT* mRNA expression may be useful for the diagnosis of NSCLC and may be an independent prognostic factor for patients with NSCLC. Nevertheless, in that study *hTERT* mRNA was detected by an *in situ* hybridization in 146 formalin fixed, paraffin embedded tissues. They found a significant correlation between *hTERT* mRNA expression, pathologic tumor status (pT), and the clinical stage, but similarly to our results, in their group no significant correlation between *hTERT* mRNA expression and age, gender, pathologic lymph node status (pN), histology, or tumor differentiation was revealed (Fujita *et al*., 2003). Surprisingly, in our study a significant difference in *hTERT* expression between two types of histopathological pattern (adenocarcinoma and SCC) was detected ($p=0.01$) (Table 2). A similar difference was not found in the other authors' studies. This observation needs to be verified in a much larger cohort of patients diagnosed with these two histological types of NSCLC. However, the percentages of samples with *hTERT* expression among non-cancerous tissues in our group are much higher than in other authors' studies (Hara *et al*., 2001; Hashim *et al*., 2011; van den Berg *et al*., 2010). This may result from the presence of epithelial cells in the samples of lung tissue. It is known that in normal adult tissues telomerase activity appears to correlate well with the differentiation stage of a cell as well as with its potential to act as a stem cell on appropriate stimulation. Telomerase expression correlates with self-renewal potential in many cell types including epithelial cells (Driscoll *et al*., 2000).

In our study, based on information about the patients' dates of death the 1-, 2-, and 5-year survival rates were calculated and then correlated with *hTERT* expression. No significant differences in *hTERT* expression in tumor tissues between deceased and living patients one, two, and five years after surgery were found (Table 2). Although numerous studies associate *hTERT* overexpression with poor prognosis (Fujita *et al*., 2003; Marchetti *et al*., 2002; Wang *et al*., 2002), other have failed to demonstrate any prognostic impact of this factor for NSCLC patients' survival rate (Kumaki *et al*., 2001; Lu *et al*., 2004). Marchetti *et al*. have shown that *hTERT* expression levels are strongly correlated with a reduced probability of survival in stage I NSCLC patients (Marchetti *et al*., 2002), while Lu and coworkers observed a lack of prognostic significance for *hTERT* mRNA expression in resected stage I NSCLC patients (Lu *et al*., 2004). Moreover, Metzger and coworkers have demonstrated a survival benefit for NSCLC patients with high *hTERT* mRNA expression (Metzger *et al*., 2009). The explanation of molecular processes resulting in such an effect remain to be discovered. Another problem is that the total cellular RNA was extracted from lung tissues which contain epithelial cells exhibiting telomerase expression; therefore, it is not possible to know whether telomerase activity is coming from the tumor cells in the sample. To overcome this limitation, a quantitative analysis of gene expression in microdissected tumor tissue would be required (Specht *et al*., 2001). However, this technique cannot be widely used in hospital practice because of high costs and lack of access to a highly specialized laboratory that applies molecular biology techniques.

Despite the unquestionable role of telomerase in the biology of immortalized cancer cells, its relationship with clinical parameters is not completely understood. Perhaps increasing data on observations linking molecular biology methods with clinical diagnostics, therapy monitoring procedures and survival rate analyses will create a sufficient base for further meta-analyses. Our study may serve as another observation in the discussion of the role of these parameters in NSCLC.

CONCLUSION

We have shown the association between *hTERT* mRNA expression and histological type of the cancer. Based on this, we concluded that *hTERT* mRNA detection by QRT-PCR can be used as a diagnostic marker in patients with NSCLC.

hTERT mRNA expression in NSCLC specimens does not correlate with survival rates and seems not to be a prognostic factor.

Acknowledgements

This study was supported by the grant from Medical University of Silesia (KNW-1-061/N/6/0).

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