ANTICANCER RESEARCH *37*: 6953-6958 (2017)
doi:10.21873/anticanres.12161

Predictive Significance of Thymidylate Synthase Expression in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: To date, many studies have suggested that thymidylate synthase (TS) could be used as a prognostic and predictive marker in non-small cell lung cancer (NSCLC) patients. However, results have been contradictory. The aim of this study was to evaluate TS mRNA levels in tumor tissue of NSCLC patients who underwent complete surgical resection and to analyze its prognostic and predictive potential. Materials and Methods: The study group consisted of 64 patients who underwent curative lung resection. Paired lung tissue samples were taken directly from the tumor tissue and from adjacent, histologically cancer-free lung tissue. The quantitative estimation of TS expression was performed by reverse transcription real-time polymerase chain reaction (RT-qPCR). The relationship between TS expression level and disease-free interval (DFI) and overall survival (OS) was analyzed. Results: There was significantly higher TS expression in NSCLC tumor tissue comparing to normal lung tissue. In the group of patients who received adjuvant chemotherapy based on platinum derivatives in combination with paclitaxel or gemcitabine, we found shorter DFI (p=0.0473) and OS (p=0.0053) in those with high expression of TS. Conclusion: Our results demonstrated the relationship of high tumor tissue TS levels to adverse prognosis in patients undergoing adjuvant chemotherapy. TS is a non-specific tumor marker with respect to NSCLC, therefore we think that its best use would be as a member of the panel of predictors of adjuvant treatment efficacy.*

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Key Words: Thymidylate synthase, NSCLC, prognosis.

Lung cancer is one of the most frequently occurring malignant neoplasms and the leading cause of death worldwide (1). About 80% of lung cancers can be classified as non-small cell lung cancer (NSCLC). NSCLC include three main histological subtypes: adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma.

Regardless of years of research, prognosis of the disease still remains unsatisfactory. Curative treatment is usually possible only in the early stages, when it is represented by surgical resection. However, surgical resection might offer a survival benefit also for patients with advanced stages (2). Excluding stage I the resection is frequently followed by adjuvant chemotherapy.

Current research in NSCLC treatment focused on increase of response to therapy. Usual therapeutic schemes contained platinum-based derivatives and antimetabolites of folic acid in different combinations. The therapeutic benefit of such therapy is limited by ability of tumor cells to overcome cytotoxicity by repairing damaged DNA. This effect can be reached by overexpression of DNA repair genes and enzymes involved in nucleic acid metabolism which provide building blocks for DNA replication and repair.

Thymidylate synthase (TS) is an essential enzyme in *de novo* biosynthetic pathway of the deoxythymidylate (dTMP). Thymidylate synthase catalyzes the reductive methylation of dUMP (deoxyuridine-5'-monophosphate or deoxyuridylate) to dTMP (deoxythymidine-5'-monophosphate or deoxythymidylate) using N^5,N^{10}-methylenetetrahydrofolate as a cofactor. The maintenance of dTMP pool is crucial for DNA replication and repair. Therefore, TS has been studied for years as a target for cancer chemotherapeutic agents: 5-fluorouracil (5-FU), 5-fluoro-2'-deoxyuridine, and folate analogs (3).

Table I. *Clinicopathological characteristics of all studied patients with NSCLC (n=64).*

Characteristic	Men n=48	Women n=16	All	
			n=64	100%
Age (years)				
<55	6	6	12	19%
55-65	28	10	38	59%
>65	14	0	14	22%
TNM - T				
1	13	4	17	27%
2	29	11	40	63%
3	6	0	6	9%
4	0	1	1	2%
TNM - N				
0	32	11	43	67%
≥1	16	5	21	33%
TNM - M				
0	48	16	64	100%
1	0	0	0	0%
Clinical stage				
I	28	11	39	61%
II	13	2	15	23%
III	7	3	10	16%
IV	0	0	0	0%
Histology				
Adenocarcinoma	15	8	23	36%
SCC	30	7	37	58%
Others	3	1	4	6%
Smoking status				
Smokers	18	11	29	45%
Non-smokers	1	2	3	5%
Ex-smokers	29	3	32	50%

SCC: Squamous cell carcinoma.

Table II. *Clinicopathological characteristics of a subgroup of patients who received chemotherapy (n=40).*

Characteristic	Men n=29	Women n=11	All	
			n=40	100%
Age (years)				
<55	4	5	9	23%
55-65	17	6	23	57%
>65	8	0	8	20%
TNM - T				
1	4	2	6	15%
2	21	9	30	75%
3	4	0	4	10%
4	0	0	0	0%
TNM - N				
0	17	7	24	60%
≥1	12	4	16	40%
TNM - M				
0	29	11	40	100%
1	0	0	0	0%
Clinical stage				
I	14	7	21	52%
II	11	2	13	33%
III	4	2	6	15%
IV	0	0	0	0%
Histology				
Adenocarcinoma	8	7	15	38%
SCC	19	4	23	57%
Others	2	0	2	5%
Smoking status				
Smokers	10	8	18	45%
Non-smokers	1	2	3	8%
Ex-smokers	18	1	19	47%

SCC: Squamous cell carcinoma.

To date, many studies have suggested that the *TS* gene as well as protein expression could be used as prognostic marker for different types of cancers including lung cancer (4-8), however, the results have been conflicting. In NSCLC, some of the studies demonstrated a correlation between high *TS* expression and poorer patient prognosis (8), whereas others showed a correlation between low *TS* expression and poor outcome (7) or found no association between *TS* expression and prognosis (9). A recently published meta-analysis concluded that prognostic value of *TS* expression needs further validation (10).

The aim of our study was to evaluate *TS* mRNA levels in tumor tissue of NSCLC patients who underwent complete surgical resection and to analyse its prognostic and predictive potential.

Materials and Methods

Patients. The studied group included 64 patients with NSCLC (48 men, 16 women), who had undergone curative lung resection between November 2004 and May 2007 at the Department of Surgery, University Hospital in Pilsen. The median age was 60.7 years at the time of surgery (range=42.8-77.7 years). Patient and tumor characteristics (distribution according to TNM, stage of disease and histology) are in Table I. Forty patients were indicated to adjuvant chemotherapy at the Department of Pneumology and Phthisiology, University Hospital in Pilsen, between 2005 and 2007. Clinicopathological data of this subgroup are listed in Table II. Postoperative adjuvant chemotherapy was indicated according to the current Guidelines of ASCO (American Society of Clinical Oncology) 2005-2007. Adjuvant chemotherapy consisted of a combination of platinum derivate (cisplatine, or carboplatine) and mitotic inhibitor (vinorelbin, or paclitaxel) or nucleoside analog (gemcitabine, or pemetrexed). No radiotherapy was applied. The median follow up was 3.59 years. As exclusion criteria for entering the study were considered age over 85 years, other malignancy and high cardiopulmonary risk (such as chronical obstructive lung disease, condition after myocardial infarction). Informed consent was received from all research participants and the study was approved by the local research ethics committee.

Table III. *Sequences of primers and corresponding UPL probes.*

Gene	Primer	Sequence	UPL probe number	Size
TS	Forward	CCCAGTTTATGGCTTCCAGT	#43	89 nt
	Reverse	GCAGTTGGTCAACTCCCTGT		
GAPDH	Forward	AGCCACATCGCTCAGACAC	#60	66 nt
	Reverse	GCCCAATACGACCAAATCC		
HPRT1	Forward	TGACCTTGATTTATTTTGCATACC	#73	102 nt
	Reverse	CGAGCAAGACGTTCAGTCCT		

TS: Thymidylate synthase; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*: hypoxanthine guanine phosphoribosyltransferase 1.

Tissue samples and RNA extraction. Paired lung tissue samples obtained directly from the tumor tissue and from adjacent, histologically cancer-free lung tissue (control) were immediately frozen to –70˚C and stored at this temperature until usage. All the samples were histologically verified as NSCLC. Total RNA from 100 mg tissue was extracted by means of FastRNAPro Green Kit (QBIOgene, Irvine, CA, USA).

Quantitative estimation of TS mRNA expression. For quantitative estimation of *TS* gene expression, reverse transcription real-time polymerase chain reaction (RT-qPCR) method with UPL probes (Universal Probe Library, Roche, Mannheim, Germany) was used. Reverse transcription (RT) was performed from 500 ng of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and random hexamers as a primer. Assays were designed by ProbeFinder 2.45 software (Roche). The sequences of primers and corresponding UPL probes are shown in Table III. The primers were synthesized by GeneriBiotech (Hradec Kralove, Czech Republic). Transcript levels were normalized against housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine guanine phosphoribosyltransferase (*HPRT1*), and a combination of aforementioned genes as well as according to the total cellular RNA content.

Statistical analysis. Non-parametric two-sided Wilcoxon signed-rank test was used for comparing the groups (tumor tissue and normal lung tissue). Evaluation of prognostic significance was performed as analysis of maximum likelihood estimates (Cox model). Kaplan-Meier survival distribution functions based on optimal cut-off values were computed for given groups. For all statistical calculations, SAS version 9.3 statistical software (SAS Institute Inc., Cary, NC, USA) was used. The results with $p<0.05$ were considered statistically significant.

Results

We compared TS expression in normal lung tissue with NSCLC tumor tissue using 4 different types of normalization (reference genes *GAPDH, HPRT1* and their combination, moreover total RNA content) (Table IV). We found statistically significantly higher TS expression in NSCLC tumor tissue compaed to normal lung tissue when data were normalized to *HPRT1* ($p=0.0049$) as well as to total RNA content ($p<0.0001$). On the other hand we did not record any

Table IV. *Difference between TS mRNA expression in tumor and normal lung tissue. Values displayed in the Table are the ratios of the TS expression in tumor and normal lung tissue, i.e. value 1 represents equal expression, values above 1 represent higher expression in tumor tissue.*

	CLC tissue/normal lung tissue			
Reference gene	25%	Median	75%	*p*-Value
Total RNA	1.464	5.856	24.251	<0.0001*
GAPDH	0.366	0.965	2.828	0.8479
HPRT1	0.784	1.931	3.605	0.0049*
2G	0.615	1.231	3.482	0.0797
	Adenocarcinoma tissue/normal lung tissue			
Reference gene	25%	Median	75%	*p*-Value
Total RNA	3.482	5.464	13.454	0.0106*
GAPDH	0.659	1.464	3.605	0.3755
HPRT1	1.515	2.297	3.138	0.0079*
2G	1.148	1.681	3.482	0.0305*
	Squamous cell carcinoma tissue/normal lung tissue			
Reference gene	25%	Median	75%	*p*-Value
Total RNA	3.607	7.031	27.416	0.0033*
GAPDH	0.309	0.720	2.231	0.4903
HPRT1	0.406	1.414	3.605	0.2366
2G	0.366	1.053	2.685	0.7083

NSCLC: Non-small cell lung cancer; *GAPDH*: thymidylate synthase expression normalised to glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*: thymidylate synthase expression normalized to hypoxanthine guanine phosphoribosyltransferase 1; 2G: normalization to geometric mean of *GAPDH* and *HPRT1*; *$p<0.05$.

Table V. *Relation between mRNA tumor tissue expression of TS (normalized against total RNA content) and disease-free interval (DFI) or overall survival (OS) in specific groups of NSCLC patients (Cox regression hazard model).*

	Expression of TS in tumor tissue			
Adjuvant chemotherapy	Parameter	HR	95% CI	*p*-Value
Yes	DFI	0.858	0.738-0.998	0.0473*
	OS	0.796	0.678-0.934	0.0053*
No	DFI	1.284	0.979-1.684	0.0709
	OS	1.146	0.974-1.348	0.0998

TS: Thymidylate synthase; NSCLC: non-small cell lung cancer; AT: adjuvant chemotherapy; HR: hazard ratio; CI: confidence interval. *$p<0.05$.

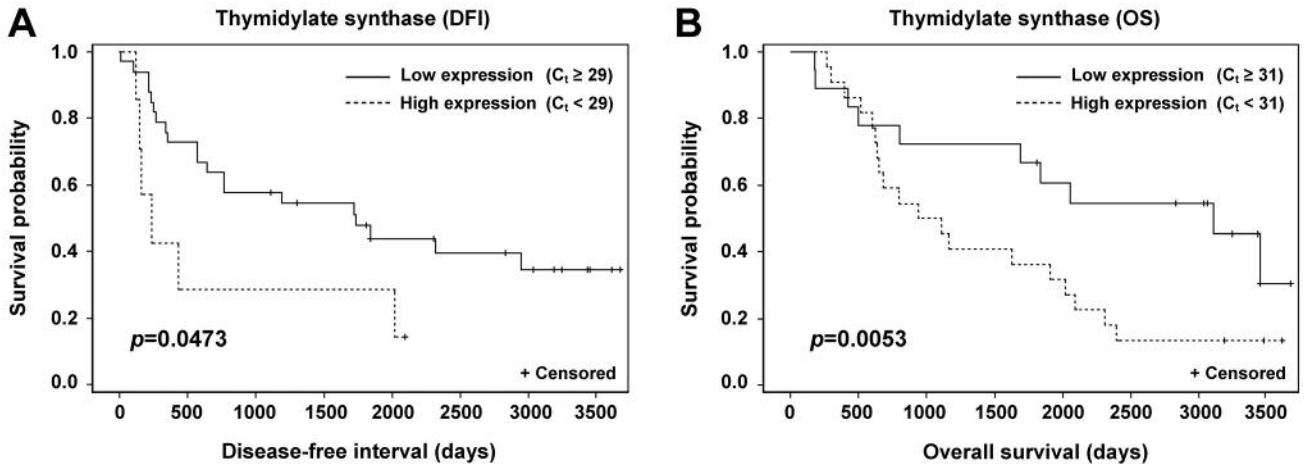

Figure 1. *Kaplan-Meier curves showing relation of thymidylate synthase expression in tumor tissue to disease-free interval (A) and overall survival (B) of surgically treated non-small cell lung cancer patients who underwent adjuvant chemotherapy.*

significant differences in case of normalization against *GAPDH* and also when normalized against gene combination including *GAPDH*. Apart from whole group we analysed also subgroups divided according to histological subtype.

When we looked at data normalized according to the RNA content we could see statistically significant elevation of the *TS* expression levels in tumor tissue at all studied groups. Data normalized against *HPRT1*, genes combination and also according to total RNA content showed significantly higher level of *TS* transcript in adenocarcinoma tissue than in normal lung tissue (p=0.0079, p=0.0305, p=0.0106, respectively). Only data normalized according to total RNA content showed significantly higher level of *TS* transcript in squamous cell carcinoma tissue than in normal lung tissue (p=0.0033).

The data were also evaluated in the relation to time to recurrence (disease-free interval, DFI) and overall survival (OS) in the whole group of NSCLC patients as well as subgroups divided according to histological subtype, chemotherapy, smoking status and involvement of lymph nodes for all types of normalization. In data normalized against *GAPDH* and *HPRT1* we did not observe any significant results. On the other hand, we recorded significant results for data normalized to the total RNA content in the subgroup of patients who received adjuvant chemotherapy. We found shorter DFI in patients with higher expression of *TS* (p=0.0473). Furthermore, we observed shorter OS in patients with higher level of *TS* mRNA (p=0.0053). Results on relation of expression levels of *TS* to prognosis mentioned above are summarized in Table V. Kaplan-Meier survival distribution functions for OS and DFI were generated (Figure 1).

Discussion

Despite advances in therapy of NSCLC, the cornerstone of first-line chemotherapy regimens are platinum derivatives in combination with pemetrexed or gemcitabine. However, overall survival varies greatly from patient to patient, even in a homogeneous group of patients of the same stage of disease who receive the same medications. One possible cause of different response to treatment could be the interindividual differences in expression of genes involved in the effect of cytostatics. The possible use of TS as a predictor of treatment has been considered since the late 1970s (3). Although a number of papers with significant results has been published on this topic, we are still failing to find the concept of the application of this key enzyme of nucleotide metabolism for predicting prognosis and treatment response. Nevertheless, based on promising published results (10-12), we believe that research of relation of TS expression to patients outcome makes sense. It is possible that assessment of TS expression will become a part of a gene expression panel of predictors that could consist of genes relevant to NSCLC as epidermal growth factor receptor (EGFR) (13), neuron specific enolase (NSE) (14), excision repair cross-complementary group 1 (ERCC1), ribonucleotide reductase subunit M1 (RRM1) and breast cancer 1 (BRCA1) (15). It is becoming more and more evident, that gene-expression profiling is the promising approach for identification of NSCLC characteristics important for making clinical decisions (16).

In tumor tissue, TS expression can be evaluated on the protein or mRNA level. In our work, we decided to assess the level of TS expression on mRNA level. The advantage

of the quantitative determination at the RNA level is a high sensitivity of RT-qPCR, which finds its application in patients who underwent a biopsy and determination is made from a very small sample of tissue, for example formalin-fixed paraffin-embedded (FFPE) tissue.

While assessing the expression at RNA level is currently solved methodologically both in terms of instrumentation and reagents sticking point remains the way of normalization of the results (17). In our study, we chose four ways of normalization with the question of whether they can also affect the results.

We have chosen two common reference genes *GAPDH* (18) and *HPRT1* (19). Normalization was performed to total RNA, the *HPRT1* gene, the *GAPDH* gene, and to the geometric mean of *HPRT1* and *GAPDH*. In the start of qPCR, *GAPDH*, a gene encoding for one of the glycolytical enzymes, was used to be the main reference gene for quantification because of its assumed expression stability. However, *GAPDH* was found to be frequently over-expressed in cancer (20). Also in our group of patients, there was higher expression of *GAPDH* in tumor tissue compared to normal lung tissue ($p<0.0001$ normalized to total RNA, $p=0.0056$ normalized to *HPRT1*). It was even demonstrated that up-regulation of *GAPDH* expression correlates with poor prognosis in NSCLC patients (21). Therefore, we think that using *GAPDH* as a reference gene wipes out the differences in expression of TS between tumor and normal lung tissue.

We observed significant elevation of *TS* expression in NSCLC tumor tissue comparing to normal lung tissue in our group of patients only in cases when for normalization was not used *GAPDH*. This observation is along with the study of Ceppi *et al*. 2012, which indicated very high increase in *TS* transcription level in tumor compared with normal lung tissue (22). Furthermore, our data showed in our group of NSCLC patients that higher expression of *TS* correlates with poor prognosis, which is similar to what was demonstrated by Huang *et al*. 2015, who measured TS expression at protein level (23).

The results from our study also agree with the data obtained by using an on-line tool (The Kaplan Meier plotter available at http://www.kmplot.com) (24) capable to assess the effect of particular gene on survival that uses gene expression data from public databases Gene Expression Omnibus (GEO), The European Genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA) (25). Figure 2 shows the Kaplan Meier plot of the survival analysis on 1926 NSCLC patients. Patients with high TS expression had significantly shorter OS ($p<0.0001$).

Despite a relatively small cohort of patients, our results clearly demonstrated the relationship of high tumor tissue *TS* levels to adverse prognosis in patients undergoing adjuvant chemotherapy. TS is a non-specific tumor marker with respect to NSCLC, therefore we think that the best for this marker use would be as a member of the panel of predictors of adjuvant treatment efficacy.

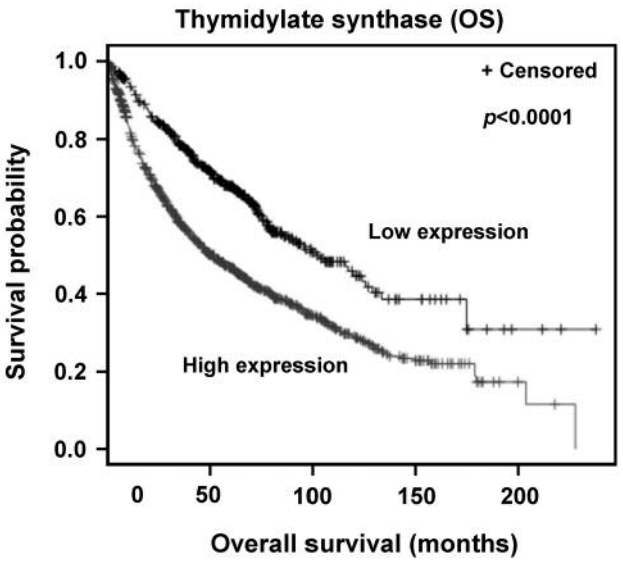

Figure 2. *Kaplan-Meier curves showing relation of thymidylate synthase expression in tumor tissue to overall survival of non-small cell lung cancer patients generated by the Kaplan Meier plotter tool.*

Conflicts of Interest

None of the Authors declares any conflict of interest.

Acknowledgements

This study was supported by the grant of Ministry of Health of the Czech Republic - Conceptual Development of Research Organization (Faculty Hospital in Pilsen - FNPl, 00669806) and the project of Faculty of Medicine in Pilsen SVV-2017-260693.

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Received August 2, 2017
Revised September 28, 2017
Accepted September 29, 2017