doi:10.4149/215_150714N389

Prognostic significance of thymidylate synthase (TS) expression in cutaneous malignant melanoma

A. SHIMIZU[1,‡,*], K. KAIRA[2,‡,*], M. YASUDA[1], T. ASAO[2], O. ISHIKAWA[1]

[1]Department of Dermatology, and [2]Department of Oncology Clinical Development, Gunma University Graduate School of Medicine, Showa-machi, Maebashi, Gunma, 371-8511, Japan

**Correspondence: shimizuakira@gunma-u.ac.jp, kkaira1970@yahoo.co.jp*
‡Contributed equally to this work.

Received July 14, 2015 / Accepted September 16, 2015

Thymidylate synthase (TS) plays an essential role in the pathogenesis and development of cancer, and TS-targeting agents have been widely used against different types of cancers. However, it remains still unclear whether or not TS is expressed in malignant melanoma. We conducted the clinicopathological study to investigate the prognostic significance of TS expression in cutaneous malignant melanoma. Ninety-nine patients with surgically resected cutaneous malignant melanoma were assessed. Tumor sections were stained by immunohistochemistry for TS, Ki-67, and microvessel density (MVD) determined by CD34. TS was positively expressed in 26% (26 out of 99). The expression of TS was significantly associated with T factor, cell proliferation (Ki-67) and MVD (CD34). By Spearman's rank test, TS expression was significantly correlated with Ki67 and CD34. By univariate analysis, ulceration, disease stage, TS, Ki-67 and CD34 had a significant relationship with survival. Multivariate analysis confirmed that TS was an independent prognostic factor for poor prognosis of cutaneous malignant melanoma.

The positive expression of TS could be a useful marker for predicting poor prognosis in patients with cutaneous malignant melanoma, and TS-targeting agents may be worth trying for the treatment of this dismal disease.

Key words: thymidylate synthase, prognosis, malignant melanoma, skin cancer

Among common skin cancers, malignant melanoma is the deadliest skin cancer [1, 2]. The therapeutic options for advanced or metastatic malignant melanoma are limited in the past decade, and the outcome after treatment is generally poor. Recently, target therapy (BRAF and MEK inhibitors) and immune checkpoint (anti-CTLA4, anti-PD-1, and anti-PD-L1) have been available for metastatic or advanced melanoma, however, each treatment has limitations. If we are able to predict outcome after any therapeutics such as surgery or systemic chemotherapy using any biomarker, any biomarkers are necessary to be identified as a significant variable. In malignant melanoma, there are no convenient biomarkers for predicting outcome after treatment. The novel biomarker to predict the outcome and response to the specific therapy should be established in patients with cutaneous malignant melanoma.

Thymidylate synthase (TS) is an enzyme that plays an important role in the DNA synthesis and catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) [3]. TS is a target enzyme of 5-fluorouracil (5-FU), which is an anticancer chemotherapeutic agent for various human cancers [4]. It has been described that the anticancer activity of 5-FU is closely associated with the intratumoral expression of TS, orotate phosphoribosyltransferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) [5]. TS is extensively expressed in various human cancers, and the increased TS expression is known to be closely associated with proliferative activity, metastasis and survival [6, 7]. We previously reported that the increased TS expression is significantly associated with tumor proliferation, angiogenesis and hypoxia in patients with lung cancer [7]. A recent review based on the analysis of 24 studies including 2280 patients has described that the cancer patients with low TS expression have statistically significantly longer overall survival (OS) and progression-free survival (PFS) than those with high TS, and cancer patients with low TS expres-

sion who received TS-targeted drugs such as pemetrexed and S-1 (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan) revealed better OS and PFS [8]. Especially, these TS-targeted drugs have been widely administered by patients with lung cancer and gastrointestinal cancer, and the chemotherapy including TS-targeted drugs are proven to be a standard regimen [9, 10]. However, it remains unclear whether TS-targeted drugs are effective for patients with advanced or metastatic malignant melanoma. A previous report described that TS gene expression was significantly higher in metastatic melanoma than in normal tissues [11]. Some experimental studies also disclosed that TS inhibitors could induce apoptosis in human melanoma cells through a caspase-mediated pathway by *in vitro* experiments [12-14]. However, there has been no study that focuses on the clinicopathological features of TS protein expression in patients with cutaneous malignant melanoma. Based on these backgrounds, we conduct the clinicopathological study to evaluate the prognostic significance of TS protein expression in patients with cutaneous malignant melanoma.

Patients and methods

Patients. We analyzed 99 consecutive patients with cutaneous malignant melanoma who underwent surgical resection at Gunma University Hospital between September 1989 and May 2011. Clinical stages were defined according to the 2009 guidelines of the American Joint Committee on Cancer (AJCC). We further analyzed 30 resected lesions with melanocytic nevi as a negative control. The authors' approach to the evaluation and resection of these tumors has been described previously [15]. This study was approved by the institutional review board of Gunma University Hospital (ethical committee for clinical studies-Gunma University faculty of Medicine).

Immunohistochemical staining. For TS, CD34, Ki-67 and p53, immunohistochemical staining was performed according to the procedures described in previous reports [6,7,15]. The following antibodies were used: a rabbit monoclonal antibody against TS (D5B3, Cell Signaling technology, 1:200 dilution), mouse monoclonal antibodies against CD34 (Nichirei, Tokyo, Japan, 1:800 dilution) and Ki-67 (Dako, Glostrup, Denmark, 1:40 dilution), and p53 (D07; Dako, 1:50 dilution). The expression of TS was considered if nuclei or cytoplasm staining was present. For TS, a semi-quantitative scoring method was used: 1= <10%, 2=10-24%, 3=25-50% and 4=51-75% of cells positive. The tumors in which stained tumor cells made up more than 10% of the tumor (score 2 or 3) were graded as positive.

The number of CD34-positive vessels was counted in four selected hot spots in a x 400 field (0.26 mm^2 field area). Microvessel density (MVD) was defined as the mean count of microvessels per 0.26 mm^2 field area. The median number of CD34-positive vessels was evaluated, and the tumours in which stained tumour cells made up more than each median value were defined as high expression. As for Ki-67, a highly cellular area of the immunostained sections was evaluated. All melanoma cells with nuclear staining of any intensity were defined as high expression. Approximately 1000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample. The median value of the Ki-67 labeling index was evaluated, and the tumour cells with greater than the median value were defined as high expression. For p53, microscopic examination for the nuclear reaction product was performed and scored, and p53 expression in greater than 10% of tumour cells was defined as positive expression [16]. The sections were assessed using a light microscopy in a blinded fashion by at least two of the authors.

Statistical analysis. Probability values of <0.05 indicated a statistically significant difference. The significance of difference was determined by Fisher's exact test. The correlation between different variables was analyzed using the nonparametric Spearman's rank test. The Kaplan-Meier method was

Table 1. Patient's demographics according to TS expression

Variables		Total	TS expression		P-value
			Positive	Negative	
		(n=99)	(n=26)	(n=73)	
Age	≤65 / > 65 yr	43 / 56	11 / 15	32 / 41	>0.999
Sex	Male / female	51 / 48	15 / 11	36 / 37	0,501
Ulceration	Yes / No	19 / 80	6 / 20	13 / 60	0,57
T factor	T1-2 / T3-4	50 / 48	4 / 22	46 / 27	**<0.001**
N factor	No / N1-2	71 / 28	17 / 9	54 / 19	0,451
Disease stage	I or II / III or IV	66 / 33	14 / 12	52 / 21	0,146
Anatomic site	Axial / Extremity	27 / 72	9 / 17	18 / 55	0,442
Tumor size	≤20 / > 20 mm	54 / 45	13 / 13	41 / 32	0,65
Ki-67	High / Low	47 / 52	21 / 5	26 / 47	**<0.001**
CD34	High / Low	54 / 45	24 / 2	30 / 43	**<0.001**
p53	Positive / Negative	73 / 26	22 / 4	51 / 22	0,196

Abbreviation: TS, thymidylate synthase.

used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. OS was determined as the time from tumour resection to death from any cause. PFS was defined as the time between tumour resection and the first disease progression or death. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using GraphPad Prism 4 software (Graph Pad Software, San Diego, CA, USA) and JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Patient's demographics. We analyzed 99 patients with cutaneous malignant melanoma. The patient's characteristics are listed in Table 1. The age of the patients ranged from 42 to 86 years, and the median age was 71 years. Most tumors (n = 94, 95%) were pathological stages I to III. Sixty patients had received postoperative adjuvant chemotherapy. The day of surgery was considered the starting day for measuring postoperative survival. A median follow-up duration for all patients was 1730 days (range, 74 to 7400 days).

Immunohistochemical analyses. The immunohistochemical analyses were performed on the 99 primary lesions of cutaneous malignant melanoma and 30 resected lesions of melanocytic nevus. Figure 1 represents the immunohistochemical staining of TS protein expression in cutaneous malignant melanoma. Positive staining of TS expression was shown in the cytoplasma and nuclei. The staining of grade 1, 2 and 3 were shown in Figures 1A, 1B and 1C, respectively. Negative staining of TS expression in melanocytic nevus was shown in Figure 1D. In 99 patients' sections of malignant melanoma, the positive expression of TS (>grade 2) was observed in 26% (26 out of 99), whereas 0% in melanocytic naevi ($P<0.001$). The cutoff points for high CD34 expression and high Ki-67 labeling index were defined as follows. The median number of CD34-positive vessels was 5 (range, 0–90), and the value of 4 was chosen as a cutoff point. The median value of the Ki-67 labeling index was 10% (range, 0–47), and the value of 10%

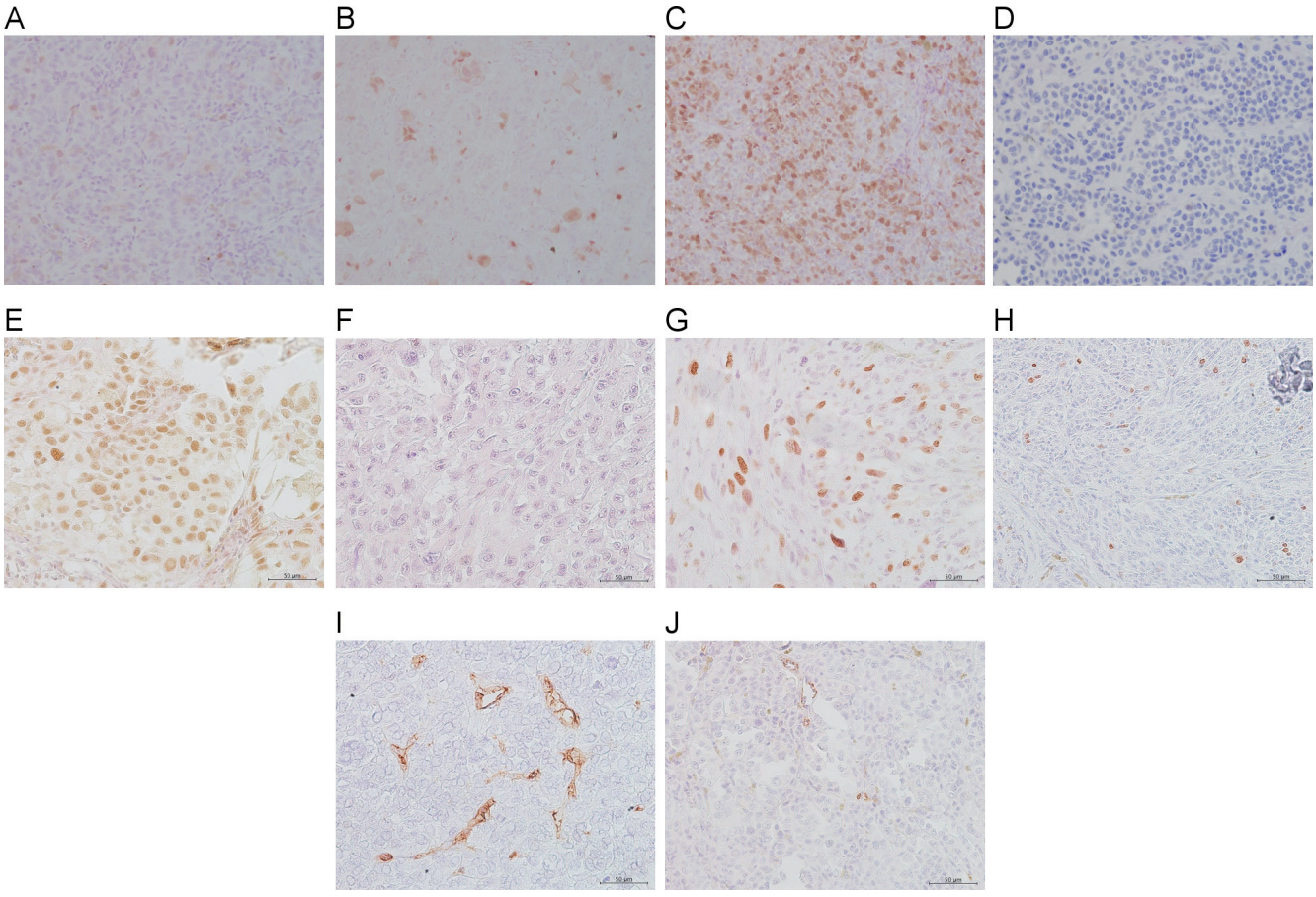

Figure 1. Immunohistochemical staining of TS expression in malignant melanoma: Positive staining of TS expression in the cytoplasma and nuclei of malignant melanoma. The score of TS immunostaining was grade 1 (A), grade 2 (B) and grade 3 (C) (hematoxylin & eosin, 200×). Negative staining of TS expression in melanocytic nevus was shown in (D) (hematoxylin & eosin, 200×). Moreover, the representative images of p53, Ki-67 and CD34 were presented: high p53 (E), low p53 (F), high Ki-67 (G), low Ki-67 (H), high CD34 (I) and low CD34 (J).

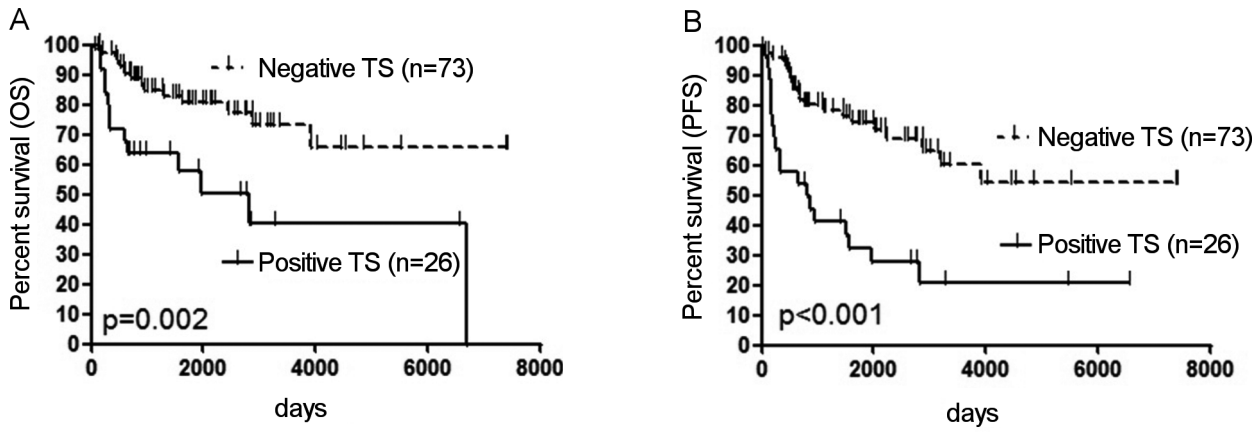

Figure 2. Outcomes stratified by Kaplan-Meier analysis of overall survival (OS) and progression-free survival (PFS) according to TS expression. A statistically significant difference in OS (A) and PFS (B) was recognized between patients with positive and negative TS expression.

was chosen as cutoff point. Positive expression of p53 was observed in 74% (73/99).

Patient's demographics according to TS expression status are listed in Table 1. The expression of TS was significantly associated with T factor, cell proliferation (Ki-67) and MVD (CD34).

Correlation between TS expression and different variables. Spearman's rank test revealed that TS expression was significantly correlated with Ki-67(r=0.474, *P*<0.001) and CD34(r=0.515, *P*<0.001), but not tumor size (r=0.174, *P*=0.085). (Table 2).

Survival analysis according to TS expression. The five-year survival rates of OS and PFS for all patients were 74% and 62 %, respectively. Of 99 patients, 28 were died and 41 had a recurrence after initial surgery. By univariate analysis, T factor, ulceration, disease stage, TS and CD34 disclosed a significant relationship with overall and progression-free survival, and Ki-67 was identified as a significant prognostic factor for PFS (Table 3). Multivariate analysis confirmed that the positive expression of TS was an independent prognostic factor for predicting worse OS and PFS after surgery. Figure 2A and 2B shows the Kaplan-Meier survival curve for OS and PFS in patients with positive and negative expression for TS.

Discussion

This is the first study that evaluate the prognostic significance of TS expression in patients with cutaneous malignant melanoma. A positive TS expression had a significant relationship with T factor, cell proliferation (Ki-67) and MVD (CD34). By univariate analysis, ulceration, disease stage, TS and CD34 had a significant relationship with survival. The positive expression of TS was identified as an independent prognostic factor for poor prognosis of cutaneous malignant melanoma.

In some experimental studies, the inhibition of TS has been described to be capable of suppressing tumor progression and inducing apoptosis in melanoma cell lines [12-14]. Buqué *et al* reported that TS-targeted agent, pemetrexed, induced DNA damage, S-phase cell cycle arrest and mitochondrial-mediated apoptosis in human melanoma cells. The possible mechanism of apoptosis was both caspase-dependent and –independent and p53-mediated [14]. In the other two reports, however, the apoptosis by TS inhibitor was considered to be mediated by a p53-independent mechanism [12, 13]. In our immunohistochemical study, the positive expression of TS was not associated with that of p53.

Several researchers have demonstrated that the high level of TS expression is closely associated with an aggressive tumor phenotype and a poor prognosis in various human neoplasms and the expression level of TS varies according to a histological type [7, 17]. The expression of TS is generally recognized to be higher in squamous cell carcinoma and small cell carcinoma than that in adenocarcinoma of the lung [6, 7, 17]. For example, the positive rate of TS protein expression yielded 51% in adenocarcinoma, 86% in squamous cell carcinoma, 96% in high-grade neuroendocrine tumor, 35% in sarcoma and 47% in mesothelioma [6]. In our study, a positive TS expression was observed in 26% of cutaneous malignant melanoma, and the expression of TS tended to be lower compared to the lung neoplasms [6].

Table 2. Correlation between TS expression and other variables

Variables	Spearman r	95% CI	*P*-value
Ki-67	0,474	0.299 to 0.618	**<0.001**
CD34	0,515	0.347 to 0.651	**<0.001**
Tumor size	0,174	-0.030 to 0.354	0,085

Abbreviation: 95%CI, 95% confidence interval.

Table 3. Univariate and multivariate survival analysis in all patients (n=99)

Variables		Overall survival					Disease-free survival				
		Univariate		Multivariate			Univariate		Multivariate		
		5-yrs rate(%)	*P*-value	HR	95% CI	*P*-value	5-yrs rate(%)	*P*-value	HR	95% CI	*P*-value
Age	≤65 / > 65 yr	81 / 69	0,215				72 / 56	0,185			
Sex	Male / female	75 / 73	0,426				64 / 60	0,419			
Ulceration	Yes / No	39 / 80	**0,006**	1.503	0.935-2.337	0,089	35 / 68	**0,005**	1.360	0.921-1.973	0,117
Disease stage	I or II / III or IV	85 / 53	**0,004**	1.461	0.970-2.204	0.069	75 / 38	**<0.001**	1.441	1.032-2.009	**0.032**
Anatomic site	Axial / Extremity	76 / 71	0.667				69 / 59	0.998			
Tumor size	≤20 / > 20 mm	84 / 62	0,169				69 / 54	0,346			
TS	Positive / Negative	57 / 81	**0,002**	1.680	1.146-2.447	**0,008**	28 / 74	**<0.001**	1.741	1.269-2.377	**<0.001**
Ki-67	High / Low	66 / 83	0,058				50 / 75	**0,016**			
CD34	High / Low	59 / 94	**<0.001**				40 / 91	**<0.001**			
p53	Positive / Negative	75 / 71	0,594				64 / 56	0,788			

Abbreviation: TS, thymidylate synthase; HR, hazard ratio; 95% CI, 95% confidence interval.

It remains unclear the relationship between the expression level of TS and the therapeutic efficacy after TS-targeted agent such as pemetrexed or S-1 in patients with cutaneous malignant melanoma. The pemetrexed and S-1 are approved in Japan for the patients with lung cancer, gastrointestinal cancer, pancreatic cancer, and head and neck cancer as daily practice. The platinum-based combination regimens including pemetrexed or S-1 have been a standard chemotherapy for patients with non-small cell lung cancer, yielding a promising efficacy and tolerable adverse events [9, 10]. Since the chemotherapeutic options for patients with advanced or metastatic malignant melanoma are limited, the new agents such as TS inhibitors should be explored as a treatment option to improve the prognosis. Further investigation is warranted to confirm the results of our study using a large-sample size and to conduct the clinical trial of TS inhibitors for malignant melanoma with high TS expression.

In a previous phase I study, it had been reported that TS-targeted drug, pemetrexed achieved a partial response against malignant melanoma [18]. Three patients with melanoma were eligible in this study, and only a few patients were treated with pemetrexed. We cannot conclude that TS-targeted drug is effective for patients with malignant melanoma. However, we believe that TS-targeted drug may have a clinical significance for the treatment of malignant melanoma, based on the results of our study and this phase I study. Further study should be conducted to investigate the efficacy of TS-targeted drugs such as pemetrexed or S-1 against patients with malignant melanoma.

Several limitations should be considered in this study. Firstly, there was not significantly relationship between disease stage and TS expression. However, a high TS expression was closely associated with T factor, cell proliferation and angiogenesis. We thought that the expression level of TS is closely correlated with the grade of tumor aggressiveness. Our study has a small sample size, therefore, this may bias the relationship between clinicopathological variables and the expression level of TS protein. Secondly, our data indicated that high TS expression was not significantly associated with lymph node metastases. As not shown in this study, 5 patients had some distant metastatic diseases. Of such patients, a high TS expression was observed in only 2 patients. As this is very small sample size with distant metastasis, it remains unclear. Further investigation is to elucidate the relationship between TS expression and metastasis in patients with malignant melanoma. Finally, we didn't investigate the relationship between the expression level of TS and the efficacy after TS targeted drugs in patients with malignant melanoma. At present, it is impossible to administer TS inhibitors in patients with malignant melanoma until approval of TS targeted agents.

In conclusion, a positive TS expression was identified as a significant independent predictor for worse outcome in patients with cutaneous malignant melanoma. TS had a close relationship with tumor proliferation, angiogenesis and aggressiveness. We should investigate whether or not the inhibition of TS could be a promising therapeutic target for patients with cutaneous malignant melanoma.

Acknowledgements: We appreciate Ms. Yuka Matsui for her technical assistance of manuscript submission and Ms. Tomoko Okada for her data collection and technical assistance. We also appreciate Dr. Madoka Kato and Dr. Ayumi Takahashi of Department Dermatology, Gunma University Graduate School of Medicine, for data collection, and Prof. Masahiko Nishiyama of Department of Molecular Pharmacology and Oncology, Gunma University Graduate School of Medicine, for the critical review of this manuscript.

References

[1] SIEGEL R, MA J, ZOU Z, JEMAL A. Cancer statistics, 2014. CA Cancer J Clin 2014;64: 9–29. http://dx.doi.org/10.3322/caac.21208

[2] LITTLE EG, EIDE MJ. Update on the current state of melanoma incidence. Dermatol Clin 2012;30: 355–361. http://dx.doi.org/10.1016/j.det.2012.04.001

[3] DANENBERG PV. Thymidylate synthase: a target enzyme in cancer chemotherapy. Biochim Biophys Acta 1997;473: 73–92.

[4] WADA H, HITOMI S, TERAMATSU T. Adjuvant chemotherapy after complete resection in non-small cell lung cancer. West Japan Study Group for Lung Cancer Surgery. J Clin Oncol 1996;14: 1048–54.

[5] NAKANO J, HUANG C, LIU D, MASUYA D, NAKASHIMA T, et al. Evaluation of biomarkers associated with 5-FU sensitivity for non-small-cell lung cancer patients postoperatively treated with UFT. Br J Cancer 2006;95: 607–15. http://dx.doi.org/10.1038/sj.bjc.6603297

[6] KAIRA K, YAMAMOTO N, ENDO M, KENMOTSU H, NAITO T, et al. ^{18}F-FDG uptake on PET is a predictive marker of thymidylate synthase expression in patients with thoracic neoplasms. Oncol Rep.2014;31: 209–15.

[7] KAIRA K, OHDE Y, NAKAGAWA K, OKUMURA T, MURAKAMI H, et al. Thymidylate synthase expression is closely associated with outcome in patients with pulmonary adenocarcinoma. Med Oncol. 2012;29: 1663–72. http://dx.doi.org/10.1007/s12032-011-0069-8

[8] LIU Q, YU Z, XIANG Y, WU N, WU L, et al. Prognostic and predictive significance of thymidylate synthase protein expression in non-small cell lung cancer: a systematic review and meta-analysis. Cancer Biomark. 2015;15: 65–78.

[9] OKAMOTO I, YOSHIOKA H, MORITA S, ANDO M, TAKEDA K, et al. Phase III trial comparing oral S-1 plus carboplatin with paclitaxel plus carboplatin in chemotherapy-naïve patients with advanced non-small-cell lung cancer: results of a west Japan oncology group study. J Clin Oncol. 2010;28: 5240–6. http://dx.doi.org/10.1200/JCO.2010.31.0326

[10] SCAGLIOTTI GV, PARIKH P, VON PAWEL J, BIESMA B, VANSTEENKISTE J, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. J Clin Oncol. 2008;26: 3543–51. http://dx.doi.org/10.1200/JCO.2007.15.0375

[11] VLAYKOVA T, JEKUNEN AP, KESOMAA M, KAIREMO KJ, PYRHONEN S, et al. Increased thymidylate synthase gene expression in metastatic melanoma. Oncology. 1997;54: 146–52. http://dx.doi.org/10.1159/000227679

[12] GIUDICE S, BENASSI L, BERTAZZONI G, COSTI MP, GELAIN A, et al. New thymidylate synthase inhibitors induce apoptosis in melanoma cell lines. Toxicol In Vitro. 2007;21: 240–8. http://dx.doi.org/10.1016/j.tiv.2006.09.023

[13] GIUDICE S, BENASSI L, BERTAZZONI G, VERATTI E, MORINI D, et al. Biological evaluation of MR36, a novel non-polyglutamatable thymidylate synthase inhibitor that blocks cell cycle progression in melanoma cell lines. Invest New Drugs. 2012;30: 1484–92. http://dx.doi.org/10.1007/s10637-011-9733-2

[14] BUQUE A, MUHIALDIN JSH, MU-OZ A, CALVO B, CARRERA S, et al. Molecular mechanism implicated in Pemetrexed-induced apoptosis in human melanoma cells. Mol Cancer. 2012;11: 25. http://dx.doi.org/10.1186/1476-4598-11-25

[15.] SHIMIZU A, KAIRA K, KATO M, YASUDA M, TAKAHASHI A, et al. Prognostic significance of L-type amino acid transporter 1 (LAT1) expression in cutaneous melanoma. Melanoma Res 2015;25: 399–405. http://dx.doi.org/10.1097/CMR.0000000000000181

[16] KAIRA K, ENDO M, ABE M, NAKAGAWA K, OHDE Y, et al. Biologic correlation of 2-[18F]-fluoro-2-deoxy-D-glucose uptake on positron emission tomography in thymic epithelial tumors. J Clin Oncol 2010;28: 3746–53. http://dx.doi.org/10.1200/JCO.2009.27.4662

[17] NAKAGAWA T, OTAKE Y, YANAGIHARA K, MIYAHARA R, ISHIKAWA S, et al. Expression of thymidylate synthase is correlated with proliferative activity in non-small cell lung cancer (NSCLC). Lung Cancer 2004;43: 145–9. http://dx.doi.org/10.1016/j.lungcan.2003.09.004

[18] THODTMANN R, DEPENBROCK H, DUMEZ H, BLATTER J, JOHNSON RD, et al. Clinical and pharmacokinetic phase I study of multitargeted antifolate (LY231514) in combination with cisplatin. J Clin Oncol. 1999;17: 3009–16.