Tohoku J. Exp. Med., 2017, **242**, 303-316

Higher Tissue Levels of Thymidylate Synthase Determined by ELISA Are Associated with Poor Prognosis of Patients with Lung Cancer

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Thymidylate synthase (TS) is essential in thymidylate biosynthesis and DNA replication. Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme in pyrimidine catabolism and is important in catabolism of 5-fluorouracil (5-FU). The significance of TS and DPD expressed in lung cancer remains controversial. Here we analyzed the relationship between TS and DPD expression and clinicopathological features of lung cancer. Enzyme-linked immunosorbent assays (ELISAs) were used to measure TS and DPD levels in paired tumor and non-tumor lung tissues obtained from 168 patients (107 adenocarcinomas, 39 squamous cell carcinomas, and 22 others), who had operations at the Shinshu University Hospital from 2004 to 2007 and were followed up for a median of 57.0 months. TS and DPD expression levels were higher in tumor tissues, and TS expression levels were significantly lower in adenocarcinomas than those in other subtypes. In addition, patients with low TS levels survived longer compared with patents with high TS levels. By contrast, DPD expression levels were not correlated with overall patient survival. Importantly, patients with low TS and DPD levels exhibited significantly prolonged survival than those with high TS and DPD. Among the 168 patients, 59 patients were treated with tegafur-uracil (UFT), a DPD-inhibitory fluoropyrimidine, and the UFT-treated patients with high TS and high DPD levels showed worst prognosis. Our study demonstrates a significant correlation between low TS expression levels and long-term prognosis of patients with lung cancer. Thus, ELISA is a clinically useful method to measure TS and DPD expression in lung cancer tissues.

Keywords: dihydropyrimidine dehydrogenase, ELISA, fluoropyrimidines, lung cancer, thymidylate synthase
Tohoku J. Exp. Med., 2017 August, **242** (4), 303-316.

Introduction

Primary lung cancer is the most frequent cause of cancer-related deaths worldwide. In Japan, primary lung cancer is the leading cause of cancer-related deaths among men and the second leading cause among women. Although the survival of patients with lung cancer has improved gradually, patient prognosis remains far from favorable. Therefore, appropriate molecular biomarkers to stratify patients according to risk of recurrence as well as effective adjuvant treatment are required.

Thymidylate synthase (TS) is a highly conserved enzyme that is essential for cell survival due to its major role in thymidylate biosynthesis and DNA replication (Carreras and Santi 1995). As the methylation reaction that TS catalyzes provides the sole *de novo* source of thymidylate within the cell, TS expression is usually upregulated in cells that are actively proliferating, such as cancer cells (Derenzini et al. 2002; Nakagawa et al. 2004). Consequently, TS is a major target of cytotoxic agents. In fact, there are many reports that elevated TS expression is associated with poor prognosis in patients with colorectal, gastric, breast, renal cell, or bladder cancer (Johnston et al. 1994; Pestalozzi et al. 1997; Lenz et al. 1998; Yamachika et al. 1998; Ishikawa et al. 1999; Edler et al. 2000; Romain et al. 2000; Mizutani et al. 2001, 2003; Popat et al. 2004).

Previous studies of lung cancer indicate that high TS expression is associated with poor prognosis in patients with non-small-cell lung cancer (NSCLC) who did not receive additional chemotherapy or radiation (Nakagawa et al. 2002; Shintani et al. 2003; Hashimoto et al. 2006; Zheng et al. 2008; Huang et al. 2015). However, some studies have failed to demonstrate a significant association between TS levels and prognosis or clinicopathological features in

Received January 23, 2017; revised and accepted July 27, 2017. Published online August 24, 2017; doi: 10.1620/tjem.242.303.
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patients with NSCLC (Higashiyama et al. 2001; Miyoshi et al. 2005).

Dihydropyrimidine dehydrogenase (DPD) is the initial rate-limiting enzyme in pyrimidine catabolism. DPD is important in catabolism of not only the naturally occurring pyrimidines (uracil and thymine) but also the widely used cancer chemotherapy agent 5-fluorouracil (5-FU). To increase the bioavailability and efficacy of 5-FU, DPD-inhibitory fluoropyrimidines have been developed. Tegafur-uracil (UFT) is a first-generation DPD-inhibitory fluoropyrimidine (Fujii et al. 1978, 1979). Although DPD expression has been measured along with that of TS in lung cancer, the association between DPD expression and clinicopathological characteristics of lung cancer has varied (Higashiyama et al. 2001; Miyoshi et al. 2005; Zheng et al. 2008). Thus, the significance of TS and DPD expression in lung cancer remains controversial.

One factor behind the controversy is the methods used to measure TS and DPD expression. In most studies that examined the association between patient prognosis and TS and DPD expression levels in lung cancer, immunohistochemistry and/or real-time polymerase chain reaction (RT-PCR) were used to evaluate TS expression in the tumor. However, whether expression levels measured by RT-PCR or immunohistochemistry reflect the enzymatic activity of TS in the tumor accurately remains uncertain. Enzyme-linked immunosorbent assay (ELISA) is a well-known method for direct quantitation of a given protein and has been used widely to measure proteins in the clinic. In fact, ELISA was used to measure TS and DPD expression levels in lung cancer (Chujo et al. 2006; Tsuchida et al. 2009). Here, we examined whether TS and DPD expression levels as determined by ELISA in clinical specimens are associated with clinicopathological factors and prognosis in patients with lung cancer.

Patients and Methods

Patients and clinical specimens

This study was conducted according to the ethical guidelines of the Declaration of Helsinki, and specific approval was obtained from the Ethics Committee of Shinshu University School of Medicine (Permit Number: 434). Patients gave their written informed consent for providing specimens for the study, and the Ethics Committee approved this consent procedure. The specimens studied were obtained from 168 patients with lung cancers who underwent operations at the Shinshu University Hospital from April 2004 to December 2007. Paired samples of tumor and non-tumor lung tissues were obtained from each resected specimen, snap-frozen in liquid nitrogen, and stored at −80°C until use. The median follow-up period after operation was 57.0 months (range, 0.3-132.6 months).

The clinicopathological characteristics of the patients are summarized in Table 1. There were 109 men (65.4%) and 59 women (34.6%), ranging in age from 35 to 89 years old (mean, 69.5 ± 9.7 years and median, 71 years). One hundred and seven patients were former or current smokers, and 61 were non-smokers. Histologically, there were 107 adenocarcinomas, 39 squamous cell carcinomas, and 22 other tumors, including 9 large cell carcinomas, 5 small cell carcinomas, 3 pleomorphic carcinomas, 3 carcinoid tumors, and 2 adenosquamous carcinomas. Patients were classified as pathological stage IA (n = 59), IB (n = 34), IIA (n = 8), IIB (n = 23), IIIA (n = 33), IIIB (n = 7), or IV (n = 4). The 4 stage-IV patients had brain metastases.

Among the 168 patients, 59 patients were treated with orally administered UFT at a dose of 300 mg per day for at least two years or until cancer relapse. An appropriate dose reduction or discontinuation of UFT due to adverse events was conducted in several cases. The mean treatment period was 358 days (range, 14-947 days).

Enzyme-linked immunosorbent assay

TS and DPD expression levels in lung cancer and non-tumor lung tissues were quantified using ELISA in collaboration with the Tsukuba Research Center at Taiho Pharmaceutical Co., Ltd. (Tsukuba, Japan), according to the method described in detail in a previous study (Kurebayashi et al. 2004). Briefly, anti-TS and anti-DPD polyclonal antibodies were produced using recombinant human TS and purified DPD extracted from the porcine liver, respectively. ELISA plates for TS or DPD were then prepared. Lung cancer tissues or normal lung tissues were homogenized, and protein was extracted from the tissues. The protein contents of the tissue extracts were colorimetrically determined. We then measured the amount (ng) of TS or DPD contained in one mg of protein using ELISA. TS activity in crude extracts obtained from clinical tissue was measured using [6-^{3}H]-fluorodeoxyuridine monophosphate as a substrate. DPD activity was measured using [6-^{14}C]-fluorouracil (5-FU) as a substrate. Correlations between enzymatic activity and TS and DPD protein levels measured by ELISA were previously confirmed by linear regression analysis in the laboratory (Kurebayashi et al. 2004).

Statistical analysis

The statistical significance of the differences between the groups was compared using Mann-Whitney *U* tests. Overall survival was defined as the time from the date of surgery to the date of death from any cause or to the date on which the patient was last known to be alive. Univariate and multivariate analyses of overall survival were performed with the Kaplan-Meier method using the log-rank test. All data were analyzed using Bell Curve for Excel software (version 2.00 Social Survey Research Information Co., Ltd., Tokyo, Japan). All statistical tests were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Expression of TS and DPD in tumor tissues and non-tumor tissues

TS and DPD expression levels were compared in the paired tumor and non-tumor lung tissue samples (Fig. 1). As some of the specimens were too small to analyze by ELISA, TS expression levels could not be quantitated in four tumor specimens and 12 non-tumor lung tissue samples, and DPD expression levels could not be quantitated in three non-tumor lung tissue samples. The median TS expression levels were 10.4 ng/mg in tumor tissues and 2.2 ng/mg in normal lung tissues. The median DPD expression levels were 266.0 ng/mg in tumor tissues and 105.8 ng/mg in normal lung tissues. Both TS and DPD were expressed at significantly higher levels in tumor tissues compared with those in the corresponding non-tumor lung tissues ($p <$

Table 1. Patient Characteristics.

Characteristics	TS expression level (ng/mg protein)			DPD expression level (ng/mg protein)		
	No. of patients (N = 164)	Tumor	p value	No. of patients (N = 168)	Tumor	p value
Sex						
Female	56	6.6 ± 1.9	< 0.001	59	247.5 ± 35.0	0.51
Male	108	15.5 ± 6.0		109	273.5 ± 21.5	
Age (years)						
mean 69.5 ± 9.7 (range, 35-89)						
< 70	78	9.1 ± 6.0	0.21	78	249.5 ± 24.8	0.23
≥ 70	86	11.0 ± 5.6		90	304.3 ± 27.4	
Smoking status						
Nonsmoker	58 (F 51, M 7)	6.6 ± 2.0	< 0.001	61 (F 54, M 7)	250.7 ± 31.4	0.91
Former/Current smoker	106 (F 5, M 101)	15.5 ± 6.1		107 (F 5, M 102)	272.4 ± 23.0	
Histology						
Adenocarcinoma (AD)	104 (F 45, M 59)	7.5 ± 3.5	< 0.001	107 (F 48, M 59)	301.2 ± 25.8	< 0.01
Non-adenocarcinoma	60 (F 6, M 54)	42.8 ± 9.3		61 (F 6, M 55)	234.5 ± 20.8	
Squamous cell carcinoma (SQ)	38 (F 3, M 35)	16.0 ± 8.4		39 (F 3, M 36)	238.7 ± 20.7	
Other type	22 (F 3, M 19)	23.2 ± 19.5		22 (F 3, M 19)	507.4 ± 43.4	
Large cell carcinoma	9 (F 0, M 9)	39.4 ± 36.8		9 (F 0, M 9)	242.2 ± 65.4	
Small cell carcinoma	5 (F 0, M 5)	40.8 ± 40.3		5 (F 0, M 5)	152.5 ± 18.5	
Pleomorphic carcinoma	3 (F 1, M 2)	17.2 ± 3.3		3 (F 1, M 2)	488.9 ± 109.0	
Carcinoid	3 (F 2, M 1)	3.9 ± 3.4		3 (F 2, M 1)	32.8 ± 16.9	
Adenosquamous carcinoma	2 (F 0, M 2)	23.2 ± 0.1		2 (F 0, M 2)	507.4 ± 5.8	
Differentiation of AD/SQ	142			146		
Well	80	7.1 ± 3.6	< 0.001	82	262.2 ± 25.6	0.06
Moderately/Poorly	62 (35/27)	14.5 ± 4.6		64 (36/28)	313.6 ± 31.7	
Stage						
I (IA, IB)	89	7.8 ± 2.2	0.07	93	261.8 ± 25.6	0.77
II (IIA, IIB)	31	16.0 ± 13.7		31	260.5 ± 36.9	
IIIA	33	13.1 ± 12.2		33	278.0 ± 43.0	
IIIB/IV	11	9.4 ± 33.0		11	352.0 ± 78.9	
EGFR Mutation						
Exon19 deletion or L858R	22	7.8 ± 4.2	0.032	23	280.5 ± 46.9	0.22
Wild-type	39	17.2 ± 12.6		39	311.3 ± 30.7	
Unknown	103	10.2 ± 4.3		106	241.6 ± 25.4	
Post operative status						
No recurrence	90	8.9 ± 5.9	0.93	93	249.5 ± 26.1	0.89
Recurrence	74	14.6 ± 5.7		75	280.5 ± 26.4	

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; AD, adenocarcinoma; SQ, squamous cell carcinoma; F, female; M, male.

0.01) (Fig. 1).

Comparison of TS and DPD expression levels in tumor tissues by clinicopathological characteristics

We compared TS and DPD expression levels in tumor tissues according to clinicopathological factors (Table 1 and Fig. 2). With regard to sex, TS expression levels were significantly lower in women than in men, whereas DPD expression levels were not significantly different between sexes (Fig. 2A). TS expression levels in tumors from nonsmokers were significantly lower than those in tumors from smokers, while DPD expression levels in tumors were not affected by smoking status (Fig. 2B). For pathological stage, TS expression levels were significantly lower in stage I tumors compared with stage II tumors, whereas DPD expression levels in the tumor did not differ according to pathological lung cancer stage (Table 1). With regard to the histological tumor types, TS expression levels were significantly lower in adenocarcinoma and squamous cell carcinoma compared with those in other histological types. DPD expression did not differ significantly among histological type (Table 1 and Fig. 2C). Among adenocarcinomas and squamous cell carcinomas, TS expression levels were significantly lower in well-differentiated tumors than those in moderately or poorly differentiated tumors (Table 1).

Univariate and multivariate analyses of TS and DPD expression levels in tumor tissues according to clinicopathological characteristics of the patients

Univariate analyses of TS and DPD expression levels according to clinicopathological factors are shown in Table 2. TS expression levels in the tumor were significantly

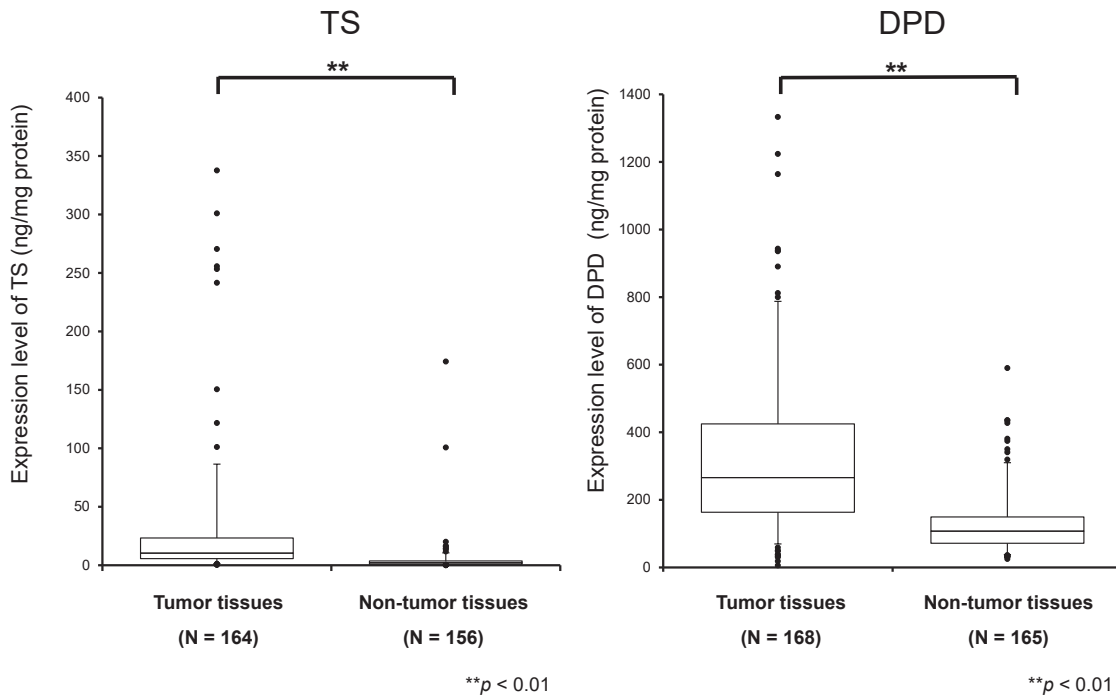


Fig. 1. TS and DPD expression levels in tumor tissues and non-tumor tissues.
The median TS expression level was 10.4 ng/mg in tumor tissues and 2.2 ng/mg in normal lung tissues. The median DPD expression levels were 266.0 ng/mg in tumor tissues and 105.8 ng/mg in normal lung tissues. Both TS and DPD levels were significantly higher in tumor tissues ($p < 0.01$).

higher in men than in women, smokers than in nonsmokers, and non-adenocarcinomas than in adenocarcinoma. On the other hand, DPD expression levels in the tumor were significantly higher in adenocarcinomas than in non-adenocarcinomas.

When these factors were entered into a multivariate model, TS expression levels were significantly lower in adenocarcinoma than in non-adenocarcinoma tissue ($p < 0.05$). In the multivariate model, DPD expression levels were significantly lower in women and non-smokers ($p < 0.001$ and $p < 0.05$, respectively).

Association between TS and DPD expression levels and clinicopathological factors in adenocarcinoma

Because TS expression levels were significantly lower in adenocarcinomas than other types of cancers, we focused on adenocarcinomas and analyzed the association between TS and DPD expression levels with clinicopathological factors in these tissues (Table 3). TS expression levels were significantly higher in men, smokers, and patients classified as N2. No significant differences in DPD expression levels were observed based on these clinicopathological factors. Neither TS nor DPD expression differed according to tumor size or Noguchi classification.

Overall patient survival and TS and DPD expression levels in tumor tissues

The median survival time was 81.4 months for all patients, with overall survival rates of 72.6% at 3 years, 58.5% at 5 years, and 35.4% at 10 years (Fig. 3A). The association between TS expression in tumors and survival is shown in Fig. 3B. When tumors were divided into two groups according to median TS expression level (10.4 ng/mg), lower TS expression levels were associated with better survival ($p < 0.01$). The association between DPD expression in tumors and survival is shown in Fig. 3C. When tumors were divided into two groups according to the median DPD expression level (264.3 ng/mg), the survival of low DPD group was slightly better than that of high DPD group. However, no statistically significant difference was observed. Moreover, when the tumors were stratified into four groups according to both TS and DPD levels, the patients with lower TS and DPD expression levels in their tumors had significantly better prognosis, compared with patients with higher TS and DPD expression levels ($p < 0.01$, Fig. 3D).

The results of a Cox proportional hazard regression analysis on overall survival in all patients with lung cancer are shown in Table 4. According to the analysis, only smoking status was an independent prognostic factor. The expression levels of TS and/or DPD were not independent prognostic factors in patients with lung cancer.

When adenocarcinomas alone were divided into four groups according to TS and DPD levels, patients with lower TS and DPD expression levels had significantly better prognoses than those with the high TS and high DPD expression levels ($p < 0.05$) (Fig. 3E). According to a Cox proportional hazard regression analysis, low TS expression and

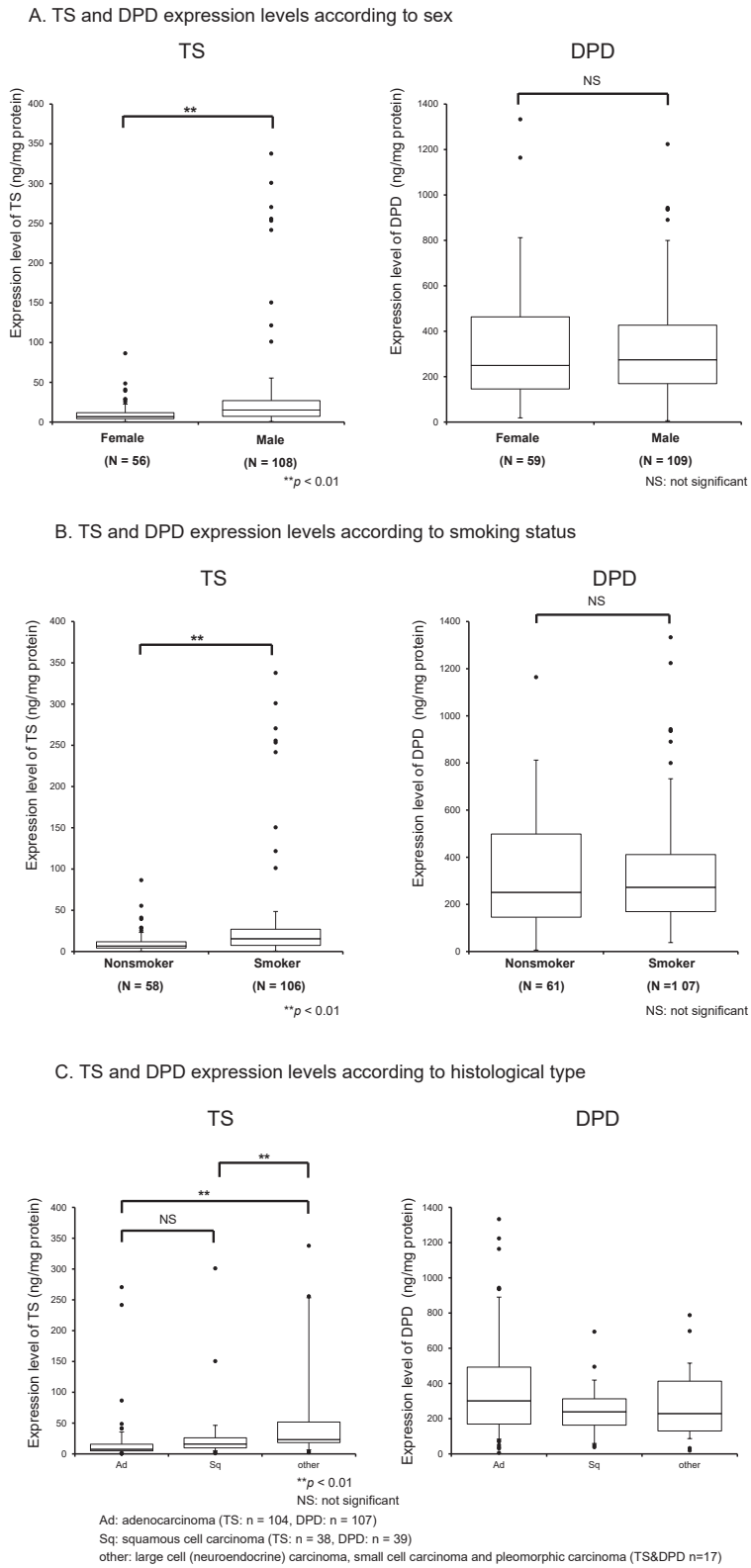


Fig. 2. TS and DPD expression levels according to clinical patient characteristics.
(A) TS and DPD expression levels according to sex. The median TS expression level in tumors was significantly lower in women, whereas the median DPD expression levels in tumors were similar between both sexes.
(B) TS and DPD expression levels according to smoking status. The median TS expression level in tumors from non-smokers (6.6 ng/mg) was significantly lower than that for smokers (15.5 ng/mg), while the median DPD expression level did not differ significantly based on smoking status.
(C) TS and DPD expression levels according to histological type. According to histological type, TS expression levels were significantly lower in adenocarcinomas and squamous cell carcinomas than those in other histological types. DPD expression levels did not differ significantly according to histological type.

Table 2. Univariate and multivariate analyses of the associations between TS and DPD expression levels in the tumor and clinicopathological features.

Characteristics	No. of patients (N = 164)	TS expression level (ng/mg protein)	p value		DPD expression level (ng/mg protein)	p value	
			Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis
Sex							
Female	56	6.6 ± 1.9	< 0.001	0.57	247.5 ± 35.0	0.51	< 0.001
Male	108	15.5 ± 6.0			273.5 ± 21.5		
Age (years)							
< 70	78	9.1 ± 6.0	0.21	0.62	249.5 ± 24.8	0.23	0.22
≥ 70	86	11.0 ± 5.6			304.3 ± 27.4		
Smoking status							
Non-smoker	58	6.6 ± 2.0	< 0.001	0.33	250.7 ± 31.4	0.91	0.046
Former/Current smoker	106	15.5 ± 6.1			272.4 ± 23.0		
Histology							
Adenocarcinoma	104	7.5 ± 3.5	< 0.001	0.02	301.2 ± 25.8	< 0.01	0.118
Non-adenocarcinoma	60	42.8 ± 9.3			234.5 ± 20.8		

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

Table 3. Association between TS/DPD expression and clinicopathological features of patients with adenocarcinoma.

Characteristics of patients	No. of patients	TS expression level (ng/mg protein)		DPD expression level (ng/mg protein)	
		Tumor	p value	Tumor	p value
Sex					
Female	49	6.3 ± 2.1	< 0.001	262.2 ± 38.2	0.30
Male	55	10.1 ± 6.3		332.5 ± 35.0	
Age (years)					
Mean 69.5 ± 10.3 (35-89)					
< 70	49	7.1 ± 5.3	0.33	273.5 ± 35.0	0.36
≥ 70	55	8.9 ± 4.6		353.0 ± 37.7	
Smoking status					
Non-smoker	49	5.7 ± 2.0	< 0.01	213.8 ± 34.1	0.35
Former/Current smoker	55	15.2 ± 6.2		346.4 ± 38.2	
Tumor size (mm)					
≦ 20	33	7.1 ± 1.8	0.38	332.5 ± 43.5	0.14
21 – 30	34	6.8 ± 2.9		262.2 ± 38.2	
> 30	37	9.9 ± 9.4		380.4 ± 51.7	
Lymph node metastasis					
N0	64	6.6 ± 1.1	< 0.05	261.8 ± 33.1	0.11
N1	17	8.3 ± 15.3		365.2 ± 51.0	
N2	23	10.6 ± 10.0		421.9 ± 56.4	
Noguchi classification	N = 31*				
Type A or B	6	5.7 ± 1.3	0.11	237.2 ± 72.8	0.19
Type C	21	7.4 ± 2.7		271.8 ± 44.7	
Type D/E/F	4	6.8 ± 5.5		440.4 ± 54.6	

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.
*Information was not available for two patients.

smoking status were independent prognostic factors in this cohort (Table 5).

Overall survival and TS and DPD expression levels in patients untreated with UFT

Among the 168 patients, 59 patients were treated with UFT as adjuvant therapy. As the treatment with UFT had a potential impact on the prognosis of the patients with lung cancer, we analyzed the associations between TS and DPD expression and survival in the cohort of patients untreated with UFT (n = 106). The association between TS expression levels in tumors and survival is shown in Fig. 4A.

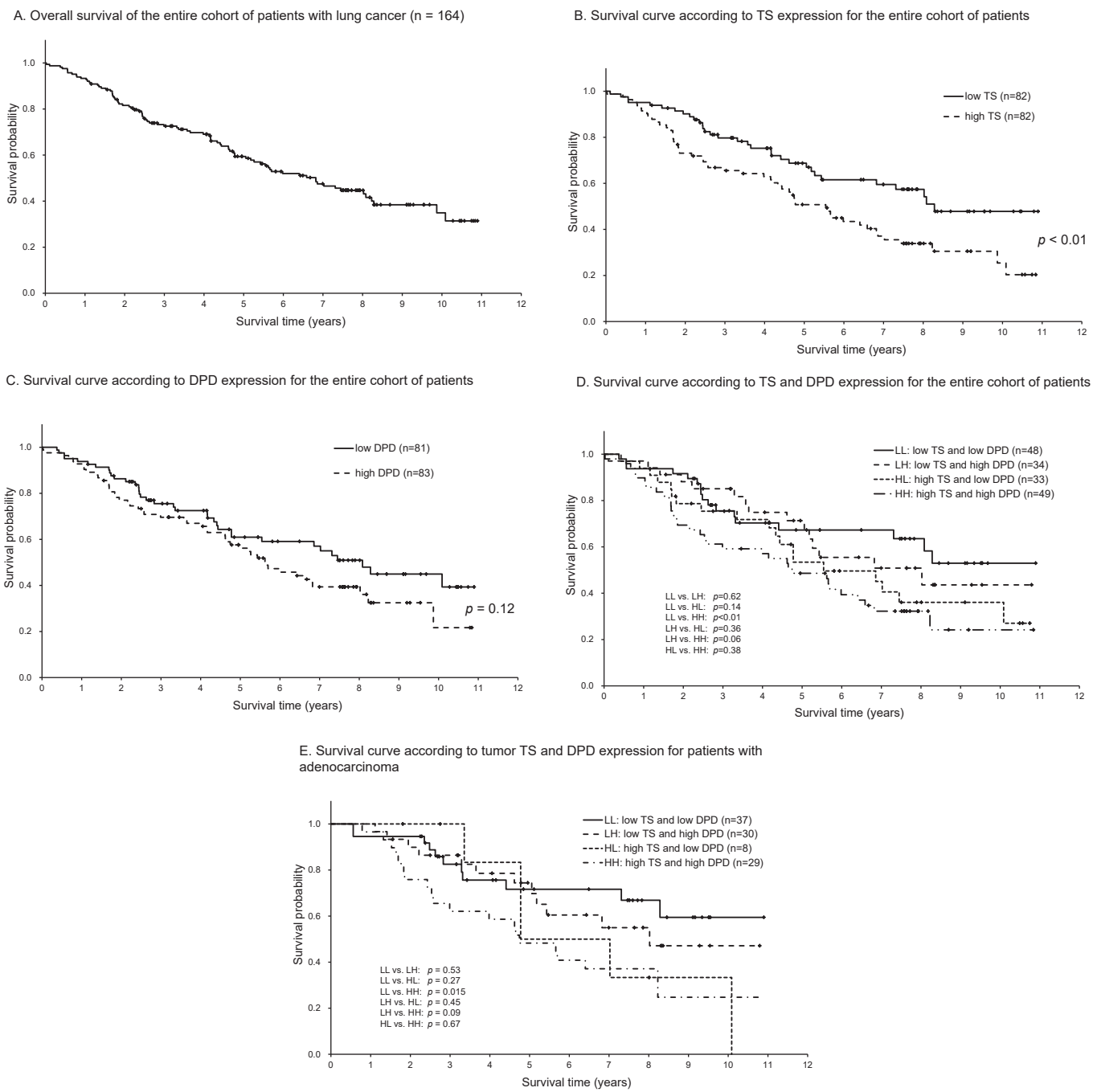


Fig. 3. Overall survival of patients according to TS and DPD expression in tumor tissues.

(A) Overall survival of the entire cohort of patients with lung cancer (n = 164). The median survival time was 41.8 months for all patients. The overall survival rates were 81.5% at 2 years, 73.8% at 3 years, and 66.7% at 4 years.

(B) Survival curve according to TS expression in tumors for the entire cohort of patients with lung cancer. Low TS expression was defined as < 10.4 ng/mg (the median expression level in tumors) (n = 82), and high TS expression was defined as ≥ 10.4 ng/mg (n = 82). Survival curve analysis shows that low TS expression is associated with significantly better survival.

(C) Survival curve according to DPD expression in tumors for the entire cohort of patients with lung cancer. Low DPD expression was defined as < 266.0 ng/mg (the median expression level in tumors) (n = 81), and high DPD expression was defined as ≥ 266.0 ng/mg (n = 83). Survival did not differ significantly according to DPD expression level.

(D) Survival curve according to TS and DPD expression in tumors from the entire cohort of patients with lung cancer. The patients were stratified into four groups according to the both TS and DPD levels in the tumor, as described above. There were 48, 34, 33, and 49 patients in the low TS and low DPD, low TS and high DPD, high TS and low DPD, and high TS and high DPD groups, respectively. Patients with low TS and low DPD expression in their tumors had significantly better prognosis than those with the high TS and high DPD expression.

(E) Survival curve according to tumor TS and DPD expression in tumors from the patients with adenocarcinoma. Patients with adenocarcinoma (n = 104) were stratified into four groups according to the both TS and DPD levels in the tumor tissues. The patients with low TS and low DPD expression in their adenocarcinoma had a significantly better prognosis than those with high TS and high DPD expression.

Table 4. Cox proportional hazards regression analysis of overall survival in patients with lung cancer.

	Hazard ratio	95% Confidence Interval			p value
Age < 70 years	1.40	0.90	-	2.19	0.14
Female	0.89	0.38	-	2.10	0.79
Non-smoker	2.59	1.07	-	6.24	0.03
Low TS expression	1.57	0.85	-	2.90	0.15
Low DPD expression	1.44	0.81	-	2.55	0.21
Low TS and low DPD expression	0.76	0.31	-	1.87	0.55

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

Table 5. Cox proportional hazards regression analysis of overall survival in patients with adenocarcinoma.

	Hazard ratio	95% Confidence Interval			p value
Age < 70 years	1.42	0.75	-	2.69	0.28
Female	0.64	0.24	-	1.72	0.38
Non-smoker	2.94	1.07	-	8.11	0.04
Low TS expression	1.01	1.00	-	1.01	0.04
Low DPD expression	1.00	1.00	-	1.00	0.58
Low TS and low DPD expression	1.20	0.51	-	2.85	0.68

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

When tumors were divided into two groups according to median TS expression level (10.4 ng/mg), low TS expression levels were associated with better survival ($p < 0.05$). The association between DPD expression levels in tumors and survival is shown in Fig. 4B. When tumors were divided into two groups according to median DPD expression level (264.3 ng/mg), no significant difference was observed between the groups, although the survival of the low-DPD group was slightly better than that of the high-DPD group. Moreover, when the tumors were stratified into four groups according to both TS and DPD levels, patients with low TS and low DPD tumor expression levels had significantly better prognoses than those with high TS and high DPD expression levels ($p < 0.05$, Fig. 4C). According to a Cox proportional hazard regression analysis, smoking status alone was an independent prognostic factor in this cohort (Table 6).

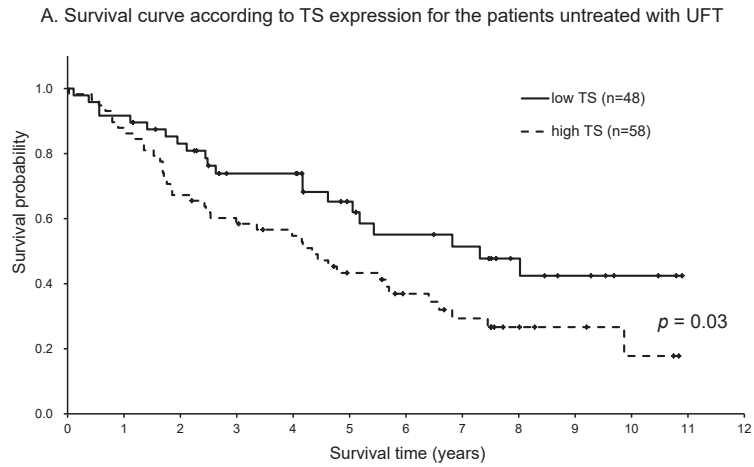
Effect of UFT on the survival of the patients with lung cancer

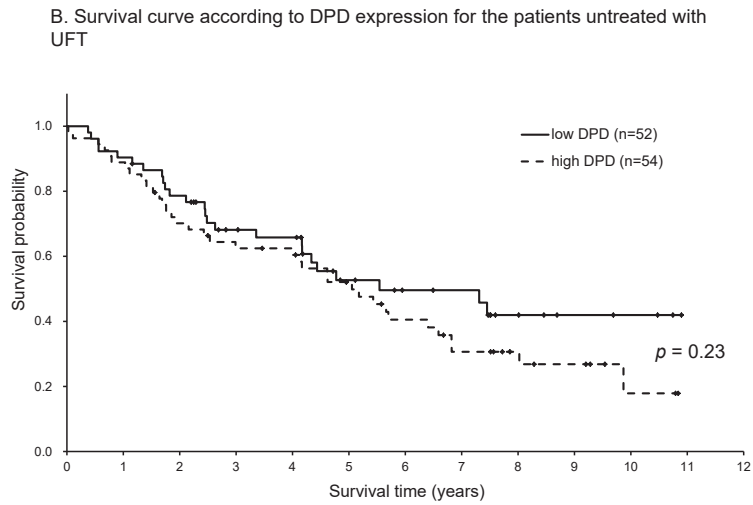
When overall survival was analyzed according to treatment with UFT, patients treated with UFT had better prognoses than those treated without UFT ($p < 0.01$) (Fig. 5). When the patients were divided into two groups according to median tumor TS or DPD expression levels, no significant differences were observed (Fig. 6A, B). When the patients were stratified into four groups according to both TS and DPD levels in their tumors, patients with high TS and DPD tumor expression levels had worse prognoses than those in other groups, although this difference was not statistically significant (Fig. 6C). According to a Cox proportional hazard regression analysis, low TS expression was an independent prognostic factor in this cohort (Table 7).

Discussion

The association between TS and DPD expression levels and clinicopathological factors in lung cancer has been controversial, as described in Introduction. Several methods, such as immunohistochemistry, RT-PCR using fresh frozen samples, gene expression analysis using micro-dissected samples from formalin-fixed paraffin-embedded specimens, and protein quantification by ELISA, have been conducted to evaluate TS and DPD activities. However, mRNA expression as determined by RT-PCR does not always represent protein expression and function (Chen et al. 2002; Zheng et al. 2008). Here we quantified TS and DPD protein levels in clinical specimens of lung cancer and corresponding normal lung tissues. We thus found that TS and DPD expression levels were increased in the tumor tissues, and TS expression in adenocarcinomas was lower than that in other carcinoma types. In addition, we demonstrated that lower TS expression levels in the tumor were associated with favorable clinicopathological characteristics, including long-term prognosis, in patients with lung cancer.

Discordance in findings on TS and DPD expression in lung cancers among studies may arise in part from the methods used to measure the expression of these enzymes. Although measurement of TS and DPD enzyme activities should be the most accurate method to determine their functions, measuring enzyme activities for TS and DPD requires a large amount of samples and radioisotope. Hence, simpler methods using small amounts of sample are needed. Tsuchida et al. (2009) reported the validity of ELISA to measure TS and DPD in lung cancer. Researchers measured the expression of TS and DPD with four different methods and demonstrated good correlations between protein expression measured by ELISA, enzyme activity, and gene expression in lung cancer tissues. Consequently, they concluded that TS and DPD levels measured by ELISA reflect enzymatic function accurately. We confirmed the correlation between protein measurements from ELISA





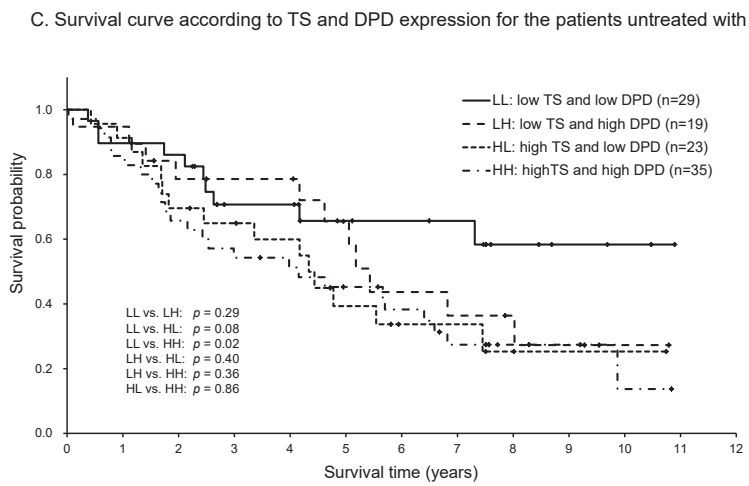


Fig. 4. Overall survival of patients not treated with UFT according to TS and DPD expression in tumors.
(A) Survival curve according to TS expression in tumors from the patients untreated with UFT. Low TS expression was defined as < 10.4 ng/mg (the median expression level in tumors) (n = 48), and high TS expression was defined as ≥ 10.4 ng/mg (n = 58). Survival curve analysis shows that low TS expression is associated with significantly better survival.
(B) Survival curve according to DPD expression in tumors from the patients untreated with UFT. Low DPD expression was defined as < 266.0 ng/mg (the median expression level in tumors) (n = 52), and high DPD expression was defined as ≥ 266.0 ng/mg (n = 54). Survival did not differ significantly according to DPD expression level.
(C) Survival curve according to TS and DPD expression in tumors from the patients untreated with UFT. The patients were stratified into four groups according to both TS and DPD levels in the tumor tissues, as described above. There were 29, 19, 23, and 35 patients in the low TS and low DPD, low TS and high DPD, high TS and low DPD, and high TS and high DPD groups, respectively. Patients with low TS and low DPD expression in their tumors had significantly better prognoses than those with high TS and high DPD expression levels.

Table 6. Cox proportional hazards regression analysis of overall survival in patients untreated with UFT.

	Hazard ratio	95% Confidence Interval			p value
Age < 70 years	1.41	0.83	-	2.41	0.20
Female sex	0.76	0.23	-	2.49	0.65
Non-smoker	3.19	0.98	-	10.35	0.05
Low TS expression	1.00	1.00	-	1.00	0.66
Low DPD expression	1.00	1.00	-	1.00	0.80
Low TS and low DPD expression	1.61	0.72	-	3.57	0.24

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

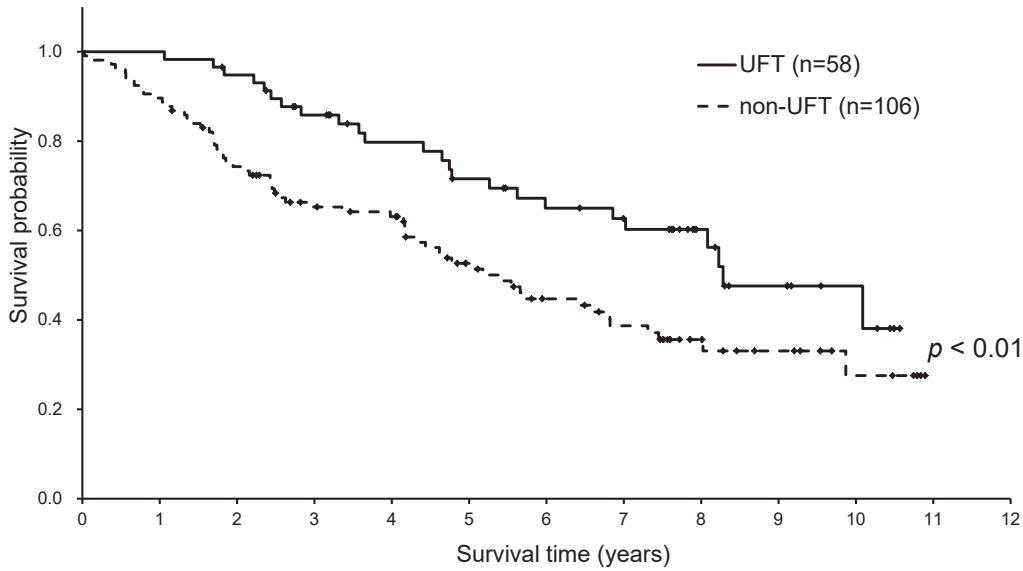


Fig. 5. Overall survival of patients according to adjuvant UFT administration.
Fifty-nine patients were treated with UFT as adjuvant therapy. Survival of the patients treated with UFT was significantly better than that of those untreated with UFT.

used and enzymatic activities of TS and DPD in clinical specimens, including those obtained from 38 patients enrolled in the study (data not shown), and differences in TS and DPD expression levels were successfully detected in the clinical specimens in our study. Although limited information has been available with regard to protein expression measured by ELISA (Nishina et al. 2004; Ma et al. 2004; Chujo et al. 2006; Tsuchida et al. 2009), our results provide further evidence that ELISA is a useful method to quantify TS and DPD activities in clinical lung cancer specimens, despite the complexity of the procedure.

TS is an essential enzyme for DNA synthesis and controls cell proliferation in a positive manner. Increased TS expression in tumor cells has been observed in several malignancies (Johnston et al. 1994; Pestalozzi et al. 1997; Lenz et al. 1998; Yamachika et al. 1998; Ishikawa et al. 1999; Edler et al. 2000; Romain et al. 2000; Mizutani et al. 2001, 2003; Popat et al. 2004). TS is involved in tumor-induced angiogenesis, and higher TS levels in tumors are associated with poor prognosis and aggressive phenotypes. In the present study, TS expression was significantly increased in tumor tissues compared with the surrounding non-tumor lung tissues. This is consistent with the results of previous studies, regardless of the methodology used to measure TS (Higashiyama et al. 2001; Shintani et al. 2003; Tanaka et al. 2011). The expression of DPD in lung cancer tissues was significantly higher than in non-tumor tissues in our study. This is also consistent with the results of previous studies on lung cancer (Otake et al. 1999; Higashiyama et al. 2001; Huang et al. 2015). Our data, together with those of previous studies, support the inhibition of DPD as a method to enhance the bioavailability and efficacy of 5-FU in lung cancer.

Tanaka et al. (2011) measured TS gene expression in laser-captured microdissected sections obtained from more than 2,500 primary lung cancers and their surrounding normal tissues using real-time RT-PCR. They observed that TS gene expression in adenocarcinomas was significantly lower than in other types of carcinoma. Furthermore, they found that TS gene expression was significantly increased with decreased tumor cell differentiation (Tanaka et al. 2011). In the present study, low TS expression levels were significantly associated with the features common for relatively slow growing adenocarcinoma, such as female sex, nonsmoker, well differentiated, and EGFR mutation. These results are thought to reflect the low activity for DNA syn-

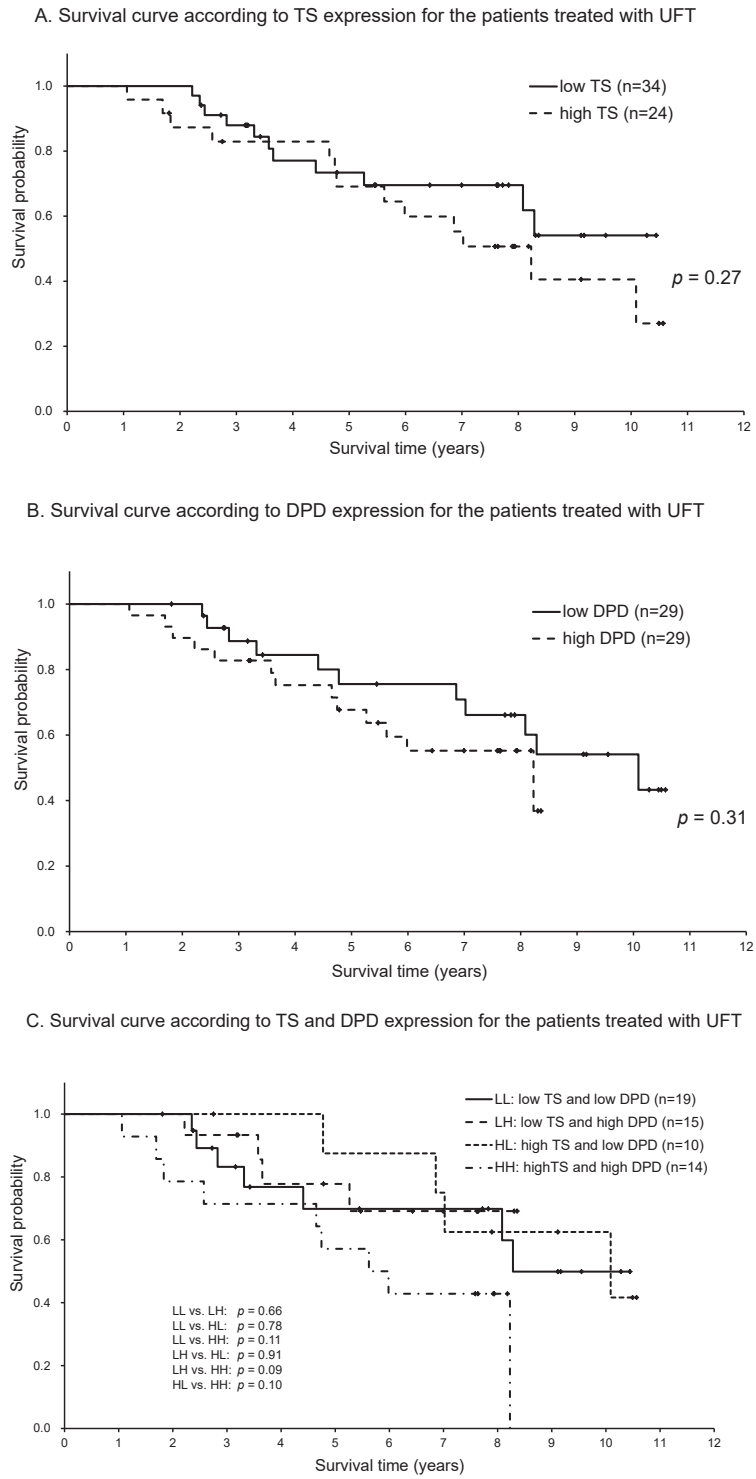


Fig. 6. Overall survival of patients treated with UFT according to TS and DPD expression in tumors.
(A) Survival curve according to TS expression in tumors from patients treated with UFT. Low TS expression was defined as < 10.4 ng/mg (the median expression level in tumors) and high TS expression was defined as ≥ 10.4 ng/mg. Survival did not differ significantly according to TS expression level in patients treated with UFT.
(B) Survival curve according to DPD expression in tumors from patients treated with UFT. Low DPD expression was defined as < 266.0 ng/mg (the median expression level in tumors), and high DPD expression was defined as ≥ 266.0 ng/mg. Survival did not differ significantly according to DPD expression in patients treated with UFT.
(C) Survival curve according to TS and DPD expression in tumors from patients treated with UFT. Patients were stratified into four groups according to both TS and DPD levels in the tumor as described above. Survival did not differ significantly among the four groups.

Table 7. Cox proportional hazards regression analysis of overall survival in patients treated with UFT.

	Hazard ratio	95% Confidence Interval			p value
Age < 70 years	1.41	0.57	-	3.49	0.46
Female sex	1.49	0.41	-	5.45	0.55
Non-smoker	1.36	0.38	-	4.93	0.64
Low TS expression	1.05	1.02	-	1.09	0.001
Low DPD expression	1.00	1.00	-	1.00	0.24
Low TS and low DPD expression	0.35	0.11	-	1.18	0.09

TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase.

thesis of these tumors. We observed the same association between TS expression and phenotype in adenocarcinomas. Our findings were consistent with RT-PCR results obtained in a large-scale study (Tanaka et al. 2011), which also supports the validity of ELISA for measuring TS expression in clinical tissues.

In the present study, TS expression levels were significantly higher in men than in women. Moreover, TS expression levels were significantly higher in smokers. Since there were not many smokers among the women in the study, we could not determine whether smoking or sex leads to high TS expression. However, some substances contained in tobacco may upregulate TS expression during lung cancer progression.

Several previous studies on lung cancer have reported that high TS expression is associated with poorer prognosis (Nakagawa et al. 2002; Shintani et al. 2003; Hashimoto et al. 2006; Zheng et al. 2008; Huang et al. 2015). Furthermore, meta-analyses have reported associations between low TS expression levels and higher objective response to TS-targeted treatments and better prognosis in patients with lung cancer (Liu et al. 2013; Wang et al. 2013; Wang et al. 2014). In the present study, lower TS expression levels were associated with favorable prognosis in the cohort of patients treated with UFT (Table 7). Moreover, our data indicate that patients with low TS expression had prolonged survival compared with those with high TS expression (Fig. 3B), while expression of DPD did not correlate with overall survival of patients with lung cancer (Fig. 3C). However, when the tumors were stratified according to combined TS and DPD expression, patients with low TS and low DPD expression survived significantly longer than those with high TS and high DPD expression (Fig. 3D). This was also true for patients with adenocarcinoma (Fig. 3E) and the cohort untreated with UFT (Fig. 4C). Therefore, the true enzymatic activity of TS, which is defined by several factors, may be an important determinant of the aggressiveness of lung cancer.

Several studies have demonstrated that the antitumor effects of 5-FU and its derivatives are associated with TS activity, which is determined by thymidine phosphatase and DPD (Beck et al. 1994; Etienne et al. 1999; Nakagawa et al. 2002). DPD-inhibitory fluoropyrimidines, such as UFT, have been developed to increase the bioavailability and efficacy of 5-FU. In fact, a randomized trial in patients with lung cancer demonstrated that postoperative adjuvant UFT chemotherapy improved survival among patients with completely resected pathological stage I adenocarcinomas (Kato et al. 2004). In the present study, the prognosis of patients treated with UFT was better than those treated without UFT. However, as this study was not a prospective randomized study, the ratio of the patients in each stage was different between the group of patients treated with UFT and that without UFT, and more patients with stage I lung cancer were included in the UFT treated group. Hence, there is a possibility that the differences in the characteristics of the patients between the two groups resulted in the better prognosis of the UFT group. On the other hand, the prognosis of patients treated with UFT did not differ according to TS or DPD expression level. Treatment with UFT may have improved the prognosis of the patients with stage I adenocarcinomas regardless of their TS protein levels. However, as the number of patients was limited in the present study, a prospective study is necessary to validate the clinical utility of TS and DPD expression measurements in lung cancer tissues by ELISA in predicting responses to TS-targeted drugs.

In conclusion, our study indicates that there is a significant correlation between TS expression as measured by ELISA and long-term prognosis in patients with lung cancer. In addition, our data suggest that the combination of TS and DPD expression levels as measured by ELISA can be used to select a favorable group of patients with lung cancer. Although further examination is required to evaluate the clinical utility of the measurement of TS and related enzymes by ELISA, our data suggest that ELISA could be an appropriate method to select patients with malignant tumors who could benefit from TS-targeted treatments, such as UFT, S-1, and pemetrexed.

Acknowledgments

We appreciate Taiho Pharmaceutical Co., Ltd. for their assistance in analysis of clinical specimens by ELISA.

Conflict of Interest

The authors declare no conflict of interest.

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