Thymidine phosphorylase affects clinical outcome following surgery and mRNA expression levels of four key enzymes for 5-fluorouracil metabolism in patients with stage I and II non-small cell lung cancer

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Abstract. The expression levels of thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), thymidylate synthase (TS) and orotate phosphoribosyltransferase (OPRT) may predict the clinical efficacy of 5-fluorouracil-based chemotherapy in patients with cancer. We herein investigated the differences in the mRNA levels of these enzymes in non-small-cell lung cancer (NSCLC) and evaluated their prognostic value for NSCLC treated by surgical resection. The intratumoral mRNA levels of *TP*, *DPD*, *TS*, and *OPRT* were quantified in 66 patients with pathological stage I and II NSCLC (adenocarcinoma or squamous cell carcinoma) following complete resection according to the Danenberg Tumor Profile method. The *TP* level was the only significant prognostic factor for disease-specific survival (DSS) following complete resection; the mean *TP* mRNA level differed significantly between the high and low mRNA expression groups. The DSS at 5 years was significantly higher in the low *TP* mRNA compared with that in the high *TP* mRNA expression group (83.4 vs. 58.6%, respectively; P=0.005). A Cox proportional hazards model revealed that pathological stage, sex, and *TP* expression were independent prognostic factors for DSS in patients with stage I and II NSCLC following complete resection. Thus, *TP* level may be used to monitor treatment efficacy and predict the outcome of NSCLC patients.

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Abbreviations: 5-FU, 5-fluorouracil; CT, computed tomography; DPD, dihydropyrimidine dehydrogenase; DSS, disease-specific survival; DTP, Danenberg tumor profile; FFPE, formalin-fixed paraffin-embedded; NSCLC, non-small-cell lung cancer; OPRT, orotate phosphoribosyltransferase; PCR, polymerase chain reaction; TP, thymidine phosphorylase; TS, thymidylate synthase; UFT, uracil and tegafur

Key words: non-small-cell lung cancer, thymidine phosphorylase, Danenberg tumor profile method, prognostic factor, complete resection

Introduction

Lung cancer remains the leading cause of cancer-related mortality in Japan, with non-small cell lung cancer (NSCLC) accounting for 87% of all cases (1); death from disease recurrence occurs in ~20% of patients with stage I and II NSCLC who underwent surgery (2). The expression levels and prognostic value of several genes have been investigated in lung cancer (3,4), but the results are inconclusive.

It is important to identify the most suitable chemotherapeutic drugs for high-risk patients following complete resection of early-stage NSCLC. Thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), thymidylate synthase (TS), and orotate phosphoribosyltransferase (OPRT) are all key enzymes in the 5-fluorouracil (5-FU) metabolic pathway and are prognostic and predictive factors in several types of cancer (5-8). The combination of uracil and tegafur (often referred to as UFT) administered orally has been shown to improve the overall survival of patients with stage IB adenocarcinoma following complete resection, and is recommended for such patients by the Japan Lung Cancer Society (9,10).

The primary aim of the present study was to investigate the association between the disease-specific survival (DSS) of patients with stage I and II NSCLC and the mRNA levels of *TP*, *DPD*, *TS*, and *OPRT*. The secondary aim was to evaluate the association between the mRNA levels of these factors and the pathological characteristics of this patient population.

Materials and methods

Patients and clinical tissue samples. Intratumoral mRNA levels were measured in 115 patients with lung cancer at Showa University Hospital (Tokyo, Japan) between January 1998 and December 2007. After excluding those with hilar or mediastinal lymph node metastasis, distant metastasis, and pulmonary metastasis, 66 patients who underwent R0 resection for pathological stage I and II NSCLC (adenocarcinoma

or squamous cell carcinoma) were enrolled in the study. All patients underwent radical lobectomy with lymph node dissection. Surgical resection is the most effective treatment for NSCLC localized in the lung. However, there are currently no criteria other than stage for selecting cases that require postoperative adjuvant therapy. In some cases, recurrence resulting in death may occur even after complete resection of NSCLC. Therefore, NSCLC patients with stage I and II disease, without lymph node, distant, or pulmonary metastasis, were selected. Pathological classification was determined according to the 7th Edition of the Union for International Cancer Control TNM Classification (11). All specimens were formalin (10%)-fixed and paraffin-embedded (FFPE), and were reviewed by pathologists at our institution. None of the patients received preoperative chemo- or radiotherapy. Blood counts, blood biochemistry tests, serum tumor marker level assessment and chest roentgenogram were performed every 2 or 3 months in the first 2 years after surgery and every 6 months in the subsequent 3 years. Furthermore, a chest computed tomography (CT) scan was performed once or twice per year. Positron emission tomography-CT, brain magnetic resonance imaging and bone scintigraphy were performed when tumor recurrence or a second primary malignancy was suspected. The protocol of the present study was approved by the Showa University Ethics Committee, and written informed consent was obtained from all participating patients.

Danenberg tumor profile (DTP) method. The DTP method (12) is used to evaluate mRNA expression levels in FFPE specimens and is superior to other methods in terms of accuracy and practicality. Standard polymerase chain reaction (PCR), biochemical assays and most other conventional techniques are impractical due to the requirement for fresh samples that are often difficult to store. In addition, other proposed methods have low accuracy and precision, since it is difficult to distinguish between cancerous stroma and normal tissues in fresh-frozen specimens. The DTP method overcomes these problems by determining the mRNA expression profiles of FFPE specimens. The procedure has been previously described in detail (13-15). The FFPE tumor specimens included in the present study were selected by an experienced pathologist following examination of hematoxylin and eosin-stained slides. Sections (10-μm) were stained with neutral fast red to visualize histological characteristics during laser capture microdissection. RNA was isolated from the FFPE specimens using a novel proprietary procedure (United States Patent Number 6,248,535; Response Genetics, Los Angeles, CA, USA). After RNA isolation, cDNA was derived from each sample as previously described (13). Quantification of the four genes of interest (*TP, DPD, TS* and *OPRT*) and an internal reference gene (*β-actin*) was performed with a fluorescence-based quantitative PCR system [ABI PRISM 7900 Sequence Detection System (TaqMan); Applied Biosystems, Foster City, CA, USA]. The PCR reaction mixture contained primers, dATP, dCTP, dGTP and dUTP, $MgCl_2$ and TaqMan buffer; the final volume of the reaction mixture, cycling conditions, primers and probes have been previously described (14). The assay yielded quantification cycle (Cq) values that were inversely proportional to the amount of cDNA in the reaction. The relative mRNA levels are expressed as the ratio (difference between Cq values) of the gene of interest and *β-actin*. Therefore, the expression level of *β-actin* mRNA in the same tissues was used as the control in our study.

Table I. Clinical characteristics of the patients.

Characteristics	No.	
Age, years		
Median (range)	68.5 (33-85)	
Sex		
Male	45	68.2%
Female	21	31.8%
Pathological type		
Adenocarcinoma	50	75.8%
Squamous cell carcinoma	16	24.2%
Pathological stage		
IA	29	43.9%
IB	26	39.4%
IIA	5	7.6%
IIB	6	9.1%
Differentiation degree		
Well differentiated	33	50.0%
Moderately differentiated	24	36.4%
Poorly differentiated	9	13.6%
Maximum tumor diameter, mm		
Median (range)	26.5 (7-80)	
Preoperative serum CEA levels, ng/ml		
Median (range)	3.4 (0.8-308.2)	
Pleural invasion		
Negative	41	62.1%
Positive	25	37.9%
Vascular invasion		
Negative	21	31.8%
Positive	45	68.2%
Lymphatic permeation		
Negative	30	45.4%
Positive	36	54.5%
UFT administration following surgery		
UFT	19	28.8%
Surgery alone	47	71.2%
Follow-up period, months		
Median (range)	76.5 (2-191)	

CEA, carcinoembryonic antigen; UFT, uracil and tegafur.

Statistical analysis. Spearman's rank correlation coefficient was used to assess correlations among the mRNA expression levels of *TP*, *DPD*, *TS* and *OPRT* and other continuous parameters. The Mann-Whitney U test or the Kruskal-Wallis test was used to compare variables, and DSS was estimated with

Table II. Association between clinicopathological variables and mRNA expression levels of four key enzymes in non-small-cell lung cancer.

Variables	Patient no.	TP levels, mean (range)	DPD levels, mean (range)	TS levels, mean (range)	OPRT levels, mean (range)
Sex		NS	NS	NS	NS
Male	45	10.23 (0.53-40.48)	1.85 (0.21-5.88)	2.26 (0.36-7.08)	1.09 (0.16-4.42)
Female	21	8.11 (3.29-25.08)	1.92 (0.20-6.15)	2.92 (0.52-18.54)	0.88 (0.22-2.95)
Age (years)		NS	NS	NS	NS
<70	35	8.59 (1.76-36.05)	1.85 (0.21-6.15)	2.14 (0.55-18.54)	0.98 (0.22-4.42)
≧70	31	10.71 (0.53-40.48)	1.89 (0.20-4.73)	2.84 (0.36-12.82)	1.07 (0.16-3.04)
Histology		NS	P<0.001	NS	P<0.001
Adenocarcinoma	50	9.19 (2.22-36.05)	2.16 (0.35-6.15)	2.15 (0.36-12.82)	0.80 (0.16-2.90)
Squamous cell carcinoma	16	10.85 (0.53-40.48)	0.95 (0.20-3.77)	3.45 (0.74-18.54)	1.72 (0.49-4.42)
Differentiation degree		NS	NS	P=0.033	NS
Well	33	7.91 (2.99-13.84)	2.20 (0.37-6.15)	1.69 (0.36-6.62)	0.84 (0.22-4.42)
Moderately	24	9.48 (0.53-25.95)	1.68 (0.20-4.64)	2.68 (0.52-7.08)	1.15 (0.16-3.04)
Poorly	9	10.13 (2.22-40.48)	1.18 (0.42-4.73)	4.76 (0.76-18.54)	1.37 (0.32-2.95)
Preoperative CEA serum level (ng/ml)		NS	NS	NS	NS
<5.0	48	9.10 (0.53-40.48)	1.98 (0.20-6.15)	2.39 (0.36-18.54)	0.94 (0.22-3.33)
≥5.0	18	10.89 (2.99-36.05)	1.83 (0.37-4.64)	2.67 (1.04-7.08)	1.06 (0.16-4.42)
Pleural invasion		NS	NS	NS	NS
Negative	41	8.71 (0.53-40.48)	2.13 (0.28-6.15)	2.44 (0.45-6.62)	0.95 (0.16-3.33)
Positive	25	10.12 (2.09-36.05)	1.44 (0.20-4.64)	2.46 (0.36-18.54)	1.07 (0.22-4.42)
Vascular invasion		P=0.042	NS	NS	NS
Negative	21	6.8 (2.09-16.83)	2.27 (0.35-4.64)	1.80 (0.36-18.54)	0.83 (0.25-3.33)
Positive	45	10.89 (0.53-40.48)	1.68 (0.20-6.15)	3.02 (0.45-7.08)	1.12 (0.16-4.42)
Lymphatic permeation		NS	NS	NS	NS
Negative	30	8.06 (1.76-24.43)	2.09 (0.21-5.88)	1.79 (0.45-7.08)	0.94 (0.16-4.92)
Positive	36	10.89 (0.53-40.48)	1.68 (0.20-6.15)	3.02 (0.36-18.54)	1.09 (0.25-2.95)

CEA, carcinoembryonic antigen; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase; OPRT, orotate phosphoribosyltransferase; NS, not significant.

the Kaplan-Meier method. The mRNA expression levels were evaluated with the DTP method, and the patients were divided into high and low expression groups according to the mean mRNA level of *TP*, *DPD*, *TS*, or *OPRT*. Differences in DSS were evaluated with a stratified log-rank test. Multivariate analyses with a Cox proportional hazards model were used to estimate the simultaneous effects of prognostic factors on DSS, which was defined as the time from surgery until death from NSCLC. Interactions of prognostic factors were also examined using the Cox proportional hazards model. JMP statistical software package, version Pro12.0 (SAS Institute, Cary, NC, USA) was used for all calculations. The level of significance was set at P<0.05. All statistical tests were two-sided.

Results

Patient characteristics. The patient characteristics are summarized in Table I. The study included 66 patients (median age, 68.5 years; range, 33-85 years), with 45 (68.2%) men and 21 (31.8%) women; 50 (75.8%) of the patients had adenocarcinoma and 16 (24.2%) had squamous cell carcinoma. Histologically, the extent of the disease ranged from stage IA to IIB (IA, n=29; IB, n=26; IIA, n=5; and IIB, n=6) and pathological N0 disease was confirmed in all patients. A total of 33 tumors were well-differentiated, 24 were moderately differentiated and 9 were poorly differentiated, and the median maximum diameter was 26.5 mm (range, 7-80 mm). The median preoperative serum level of carcinoembryonic antigen was 3.4 ng/ml (range, 0.8-308.2 ng/ml). Pleural invasion, vascular invasion and lymphatic permeation were confirmed in 25 (37.9%), 45 (68.2%) and 36 (54.5%) patients, respectively. No patient received induction chemo- or radiotherapy or molecular-targeted therapy. Postoperative adjuvant therapy included platinum-based chemotherapy in 5 patients and UFT administration in 19 patients. Tumor recurrence occurred in 28 patients (42.4%), who were then treated by platinum-based chemotherapy (n=15), chemoradiotherapy

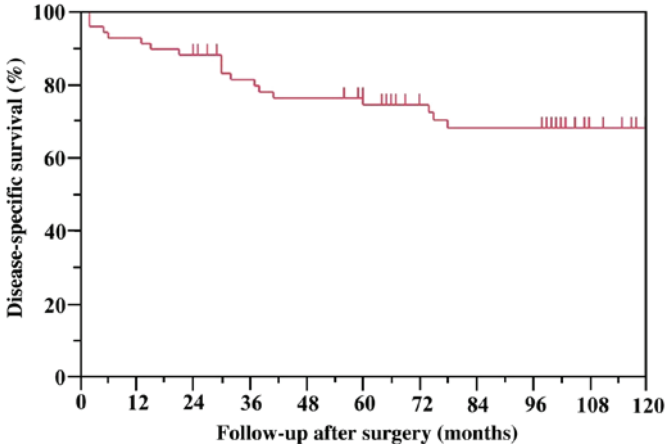

Figure 1. The DSS rate at 5 years after lung surgery was 74.2% among patients with pathological stage I and II NSCLC. DSS, disease-specific survival; NSCLC, non-small-cell lung cancer.

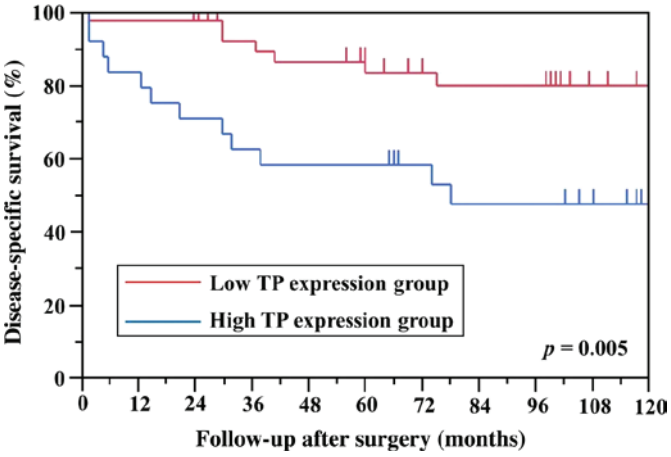

Figure 2. DSS after surgery in the high and low *TP* expression groups. Patients were divided according to mean *TP* mRNA expression value (9.03). The DSS at 5 years was higher in the low *TP* mRNA compared with that in the high *TP* mRNA expression group (83.4 vs. 58.6%, respectively; P=0.005). DSS, disease-specific survival; TP, thymidine phosphorylase.

(n=5) and surgery (n=5); the remaining 3 patients received no treatment for tumor recurrence due to their poor general condition. The median follow-up period was 76.5 months (range, 2-191 months).

The correlations between the mRNA levels of *TP*, *DPD*, *TS*, and *OPRT* genes and clinicopathological factors are shown in Table II. The mean expression levels of *TP*, *DPD*, *TS*, and *OPRT* in NSCLC patients were 9.03 (range, 0.53-40.48), 1.96 (range, 0.20-6.15), 2.45 (range, 0.36-18.54) and 0.71 (range, 0.16-4.42), respectively. *TP* levels were higher in patients with vascular invasion. *DPD* levels were negatively correlated with maximum tumor diameter (P<0.001, r=-0.553), and were higher in patients with adenocarcinoma compared with those with squamous cell carcinoma (P<0.001), whereas *OPRT* mRNA expression levels were higher in patients with squamous cell carcinoma compared with those with adenocarcinoma (P<0.001). *TS* levels were higher in patients with more poorly differentiated NSCLC (P=0.033). There was also a positive correlation between *TS* and *OPRT* levels (P<0.001, r=0.542).

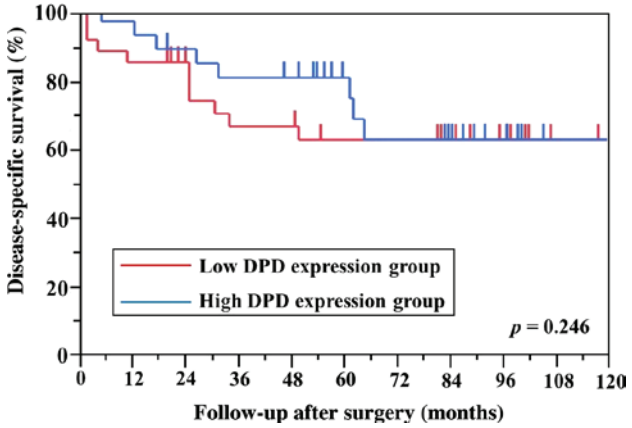

Figure 3. DSS after surgery in the high and low *DPD* expression groups. Patients were divided according to mean *DPD* mRNA expression value (1.96). There was no statistically significant difference between the high and low *DPD* expression groups (62.3 vs. 84.6%, respectively; P=0.246). DSS, disease-specific survival; DPD, dihydropyrimidine dehydrogenase.

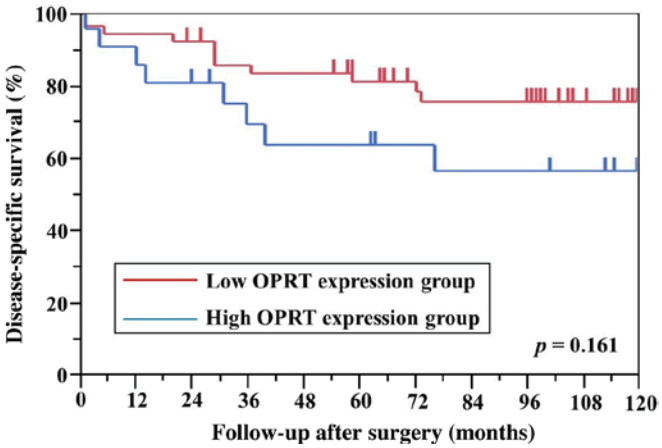

Figure 4. DSS after surgery in the high and low *TS* expression groups. Patients were divided according to mean *TS* mRNA expression value (2.45). There was no statistically significant difference between the high and low *TS* expression groups (81.1 vs. 67.0%, respectively; P=0.165). DSS, disease-specific survival; TS, thymidylate synthase.

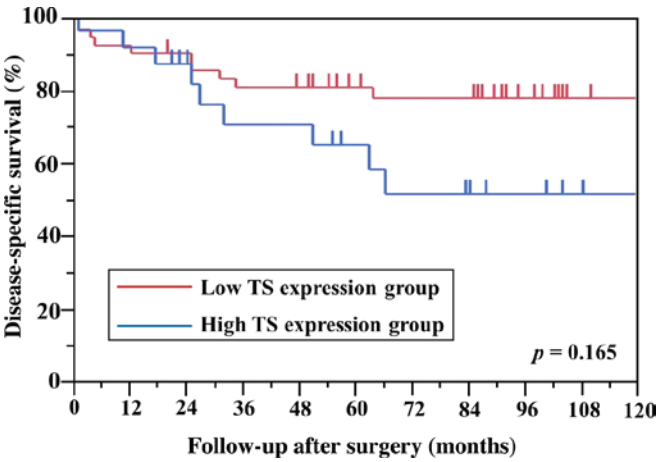

Figure 5. DSS after surgery in the high and low *OPRT* expression groups. Patients were divided according to mean *OPRT* mRNA expression value (0.71). There was no statistically significant difference between the high and low *OPRT* expression groups (81.4 vs. 66.5%, respectively; P=0.161). DSS, disease-specific survival; OPRT, orotate phosphoribosyltransferase.

Table III. Cox analysis of potential prognostic factors of DSS: Univariate and multivariate analysis.

Variables	n	5-year DSS (%)	P-value	HR	95% CI	P-value
Age (<70 vs. ≥70 years)	35/31	76.4/71.5	0.506			
Sex (male vs. female)	45/21	63.9/95.0	0.003	7.85	1.56-14.30	0.047
Adenocarcinoma vs. squamous cell carcinoma	50/16	76.9/67.7	0.144			
Pathological stage (I vs. II)	55/11	80.7/40.9	0.003	0.19	0.06-0.64	0.005
Well vs. moderately differentiated	33/24	80.6/73.5	0.218			
Moderately vs. poorly differentiated	24/9	73.5/55.6	0.386			
Well vs. poorly differentiated	33/9	80.6/55.6	0.054			
Preoperative serum CEA levels (low vs. high)	48/18	76.9/63.5	0.665			
Pleural invasion (negative vs. positive)	41/25	79.8/64.0	0.267			
Vascular invasion (negative vs. positive)	21/45	93.3/62.0	0.052			
Lymphatic permeation (negative vs. positive)	30/36	82.9/58.6	0.072			
TP (low vs. high) mean: 9.03	41/25	83.4/58.6	0.005	0.19	0.06-0.56	0.003
DPD (low vs. high) mean: 1.96	35/31	62.3/84.6	0.246			
TS (low vs. high) mean: 2.45	39/27	81.1/67.0	0.165			
OPRT (low vs. high) mean: 0.71	34/32	81.4/66.5	0.161			

DSS, disease-specific survival; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase; OPRT, orotate phosphoribosyltransferase.

The 5-year DSS rate of all 66 patients who underwent complete resection was 74.2% (Fig. 1). Among the examined genes, only *TP* levels differed significantly between the high and low mRNA expression groups for DSS (Fig. 2). There were no differences in *DPD*, *TS* and *OPRT* levels between the high and low mRNA expression groups for DSS (Figs. 3-5). A univariate analysis revealed that DSS was better in patients who were female, those with pathological stage I NSCLC and those with low *TP* mRNA levels, and the Cox proportional hazards model revealed that sex, pathological stage, and *TP* mRNA expression were independent prognostic factors for DSS (Table III).

Discussion

In the present study, the association between the expression levels of key enzymes associated with 5-FU metabolism and the pathological characteristics of patients with stage I and II NSCLC was investigated. The *TS* expression level was low in patients with well-differentiated NSCLC; *DPD* was downregulated in patients with squamous cell carcinoma, which was negatively correlated with maximum tumor diameter; *TP* was highly expressed in patients with vascular invasion; and the *OPRT* level was decreased in patients with adenocarcinoma. The reason for these findings is not known; however, it was reported that adenocarcinoma *in situ*, previously classified as bronchioloalveolar carcinoma (16), has a significantly higher epidermal growth factor receptor (EGFR) mutation frequency and *DPD* mRNA levels compared with other histological types (17), which may explain the high *DPD* mRNA levels observed in adenocarcinoma. On the other hand, OPRT activity is known to be increased in rapidly growing cells, including normal cells, such as those in the testis (18). Squamous cell carcinoma exhibited higher *OPRT* mRNA levels compared with adenocarcinoma, due to a shorter doubling time (19). TS is an enzyme that plays an important role in DNA biosynthesis and repair. Evidence from *in vitro* and *in vivo* studies indicates that TS contributes to cancer development through cell cycle regulation (20). More poorly differentiated carcinomas exhibit higher rates of tumor cell proliferation. Thus, an increased *TS* expression level in NSCLC likely reflects poor differentiation.

The observed associations between the mRNA levels of factors related to 5-FU metabolism and pathological characteristics in this study are in agreement with previous reports (21-24). *TP* mRNA level was found to be an independent prognostic factor for DSS in NSCLC. TP is a nucleoside metabolic enzyme that plays an important role in the pyrimidine salvage pathway. 5-FU is transformed into a deoxyribose fluorouracil nucleoside monophosphate through TP, which forms a complex with methylene tetrahydrofolate that inhibits TS activity, thereby interfering with DNA replication and inhibiting tumor cell growth and proliferation. DPD is the initial and rate-limiting enzyme in 5-FU catabolism. *In vivo*, >85% of 5-FU is reduced to inactive metabolites by enzymes produced in the liver and other tissues, which are then excreted by the kidneys (25).

TP catalyzes the reversible conversion of thymidine to thymine and 2-deoxy-α-d-ribose-1-phosphate, and the phosphorolysis of deoxyuridine to uracil and 2-deoxy-α-d-ribose-1-phosphate (26). A major function of TP is to control the intracellular levels of thymidine, which is cytotoxic at high concentrations and causes errors in DNA replication. Thus, TP expression is crucial for the efficacy of 5-FU-based chemotherapy (25,26).

Apart from its role as a nucleoside metabolism enzyme, TP also functions as an angiogenic factor that is identical to platelet-derived endothelial cell growth factor (PD-ECGF) (27). TP enhances angiogenesis in the tumor via two distinct mechanisms: By stimulating endothelial cell migration, and the release of angiogenic factors from malignant cells and

stromal cells into the tumor microenvironment. TP-expressing cells were shown to secrete angiogenic factors (interleukin-8, basic fibroblast growth factor and tumor necrosis factor-α) that stimulated endothelial cell migration and invasion, but not proliferation (26). It was also reported that a high level of TP caused more aggressive cancer growth with a higher incidence of vascular infiltration and metastasis in breast, colorectal and gastric cancers (28-30). PD-ECGF/TP has been implicated in the pathogenesis of NSCLC, and its upregulation defines a more aggressive tumor phenotype associated with a poor prognosis, particularly in cases without nodal involvement. However, PD-ECGF/TP expression was unrelated to the degree of differentiation, nodal status and histology, or the expression of Ki67, EGFR and p53 (31).

The 'Nottingham Prognostic Index' is an evaluation of breast cancer based on tumor diameter, lymph node metastasis, and differentiation level (32). Therefore, we have not used this score in our study, because cases with lymph node metastasis were excluded, and the tumor diameter was reflected almost exactly by pathological stage. Moreover, it is not suitable for evaluating angiogenesis.

In this study, the low *TP* expression group had better DSS compared with the high expression group in stage I and II NSCLC. The *TP* expression level was also higher in patients with vascular invasion. These results indicate that *TP* expression is a potential marker for tumor malignancy, including vascular invasion or micrometastasis. However, our study had certain limitations: adjuvant chemotherapy was administered according to the physician's preference. Moreover, we were unable to retrieve information on certain patient characteristics, such as smoking habit, other angiogenic factors, and mutational status. In addition, we did not measure the enzyme expression levels in the normal lung tissue or in the healthy tissue surrounding the lung cancer tissue. Finally, considering that an optimal cut-off value for mRNA expression level was not defined in previous studies, we considered the mean value as the cut-off value based on the following thoughts (33-36). Enzyme expression levels are presented in a quantitative manner and there are no excessive differences between the expression levels of distinct enzymes.

There have been few investigations on the utility of TP as a prognostic factor for patients with completely resectable NSCLC, whereas no studies to date have quantitatively analyzed TP expression in this patient population (31,37). Our results demonstrated that intratumoral *TP* expression is an important prognostic factor in patients with completely resected NSCLC without metastasis. Thus, additional and more powerful adjuvant therapies should be considered for early-stage NSCLC with high *TP* mRNA levels in order to improve patient outcome.

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Availability of data and materials

The data generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the design of the study and the writing of the manuscript. NH, DK, SY and MK undertook the research and performed the analyses. All authors reviewed and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

The study was conducted with the approval of the Institutional Ethics Committee at Showa University Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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