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Depolarized MUC1 Expression Is Closely Associated With Hypoxic Markers and Poor Outcome in Resected Non–Small Cell Lung Cancer

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Abstract

MUC1 is transmembrane mucin aberrantly overexpressed in various cancers. However, little is known about how MUC1 expression is associated with hypoxia, glucose metabolism, and epidermal growth factor receptor (EGFR) pathway, which are related to cancer progression. The aim of this study is to evaluate the relationship between MUC1 expression and these molecular markers in lung cancer. Of all 126 patients, high-grade polarized expression (HP), low-grade polarized expression (LP), and depolarized expression (DP) group were 50 (39.7%), 35 (27.8%), and 41 (32.5%), respectively. Depolarized MUC1 expression was significantly associated with poor outcome and was closely correlated with glucose metabolism (Glut1), hypoxia (HIF-1α), angiogenesis (vascular endothelial growth factor and microvessel density), amino acid metabolism (LAT1), and EGFR expression. High-grade polarized MUC1 expression was associated with favorable prognosis and adenocarcinoma. Depolarized MUC1 expression was significantly associated with poor outcome. Glucose metabolism, hypoxia, angiogenesis, amino acid metabolism, and EGFR pathway may play an important role in the development of depolarized MUC1 expression.

Keywords

MUC1, NSCLC, prognosis, hypoxia, EGFR, Glut1

Introduction

MUC1 is a transmembrane mucin consisting of a heavily *O*-glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic tail of 72 amino acids.[1] MUC1 oncoprotein has been documented to be aberrantly expressed at high levels in most human neoplasms and plays important roles in development and progression of malignant tumors.[2-4] Moreover, MUC1 has emerged as a target molecule in immunotherapy for various cancers. As the mechanism of a target for cancer treatment, unmasked epitopes of MUC1 core protein expressed on tumor cells have been described to be able to elicit a strong antitumor immunity.[4] Several researchers described that MUC1 expression is correlated with poor outcome in various human neoplasms.[3,5]

Non–small cell lung cancer (NSCLC) is the leading cause of cancer death and has a dismal prognosis. To improve the outcome of NSCLC patients, the biomarkers that may predict the prognosis and response to the specific therapy should be established. Tumor staging and performance status have been consistently shown to be the most powerful prognostic tool for predicting the outcome of NSCLC patients.[6] However, there has been no established clinical marker, which correlates with the response to the treatment and the prognosis, in patients with NSCLC.

Recently, several reports have documented that the overexpression of MUC1 has a crucial role on the cancer progression and metastasis, leading to poor outcome, in patients with NSCLC.[3,5,7-10] Giatromanolaki et al[9] described that MUC1 expression was significantly correlated with vascular endothelial growth factor (VEGF) expression and was found in highly vascularized NSCLC tumors. One in

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vitro study suggests that hypoxia enhances MUC1 expression through the transcriptional regulation by hypoxia-inducible factor-1 alpha (HIF-1α) in lung adenocarcinoma (AC) cell line.[11] HIF-1α is considered to support tumor growth by the induction of angiogenesis via the expression of the VEGF and also by high and anaerobic metabolic mechanisms.[12] Glucose transporter-1 (Glut1) is thought to be a possible intrinsic marker of hypoxia, and the expression of Glut1 has been found to be regulated by hypoxia in a HIF-1-dependent way.[13,14] In addition, mammalian target of rapamycin (mTOR) is a downstream component of the PI3K/Akt pathway involved in the regulation of cell proliferation, angiogenesis, and metabolism. Epidermal growth factor receptor (EGFR) is an upstream component of the PI3K/Akt/mTOR signaling pathway in human neoplasms and is overexpressed in many cancers. Hisatsune et al[15] reported that anti-MUC1 antibody inhibits EGFR signaling in cancer cell, and MUC1 is closely associated with EGFR expression. One in vitro study demonstrated that expression of MUC1 activates the PI3K/Akt pathway.[16] However, little is known about how MUC1 expression is associated with EGFR expression, Akt/mTOR signaling pathway, and hypoxic markers in human neoplasms. As many factors can influence the extent of MUC1 expression, the underlying mechanisms for the overexpression of MUC1 are still a matter of debate in patients with NSCLC. In patients with NSCLC, hypoxia, angiogenesis, glucose metabolism, amino acid metabolism, and EGFR pathway are closely associated with tumor progression, metastasis, and prognosis.[17,18] Based on these backgrounds, we conducted an immunohistochemical study to examine how MUC1 expression is correlated with hypoxic markers (Glut1, Glut3, hexokinase I, HIF-1α, VEGF, and microvessel density [MVD] determinated by CD34), EGFR pathway (EGFR, phosphatase and tensin analogue [PTEN], phospho-Akt, phospho-mTOR, and phospho-S6K), amino acid metabolism (L-type amino acid transporter 1 [LAT1]), and cell cycle regulator (p53) in patients with resected NSCLC.

Materials and Methods

Patients

Between October 2002 and May 2004, 133 consecutive patients with resectable NSCLC underwent curative resection at Shizuoka Cancer Center. Of these, 7 patients were excluded for further studies because the tissue specimens were not available. Thus, a total of 126 patients (81 men, 45 women) were eligible for the study. The study protocol was approved by the institutional review board.

The age of the patients ranged from 40 to 89 years, and the mean age was 66 years. None of the patients had received neoadjuvant chemotherapy. The tumor stage was determined by diagnostic imaging including computed tomography and 2-[^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography (2-^{18}F-FDG PET). All surgical specimens were reviewed and classified according to the World Health Organization classification by an experienced lung pathologist who was unaware of clinical or imaging findings. Pathologic tumor–node–metastasis (TNM) stages were established using the International System for Staging Lung Cancer adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer.[19] Histologically, 82 patients had AC, 37 had squamous cell carcinoma (SQC), and 7 had large cell carcinoma. Of the total patients, 63, 25, and 38 had stage I, stage II, and stage III tumors, respectively. As postoperative adjuvant therapies, platinum-based chemotherapy, radiation, and oral administration of tegafur (a fluorouracil-derivative drug) were administered to 9, 1, and 8 patients, respectively. Intraoperative therapy was not performed on any patient. The postoperative clinical course was assessed by analyzing outpatient medical records and by marking telephone inquiries. The day of surgery was considered the starting day for counting postoperative survival. The follow-up duration ranged from 6 to 88 months (median = 62 months).

Immunohistochemical Staining

Immunohistochemical staining was performed according to the procedure described in the previous reports.[3,18,20,21] The technical details of the antibodies employed in this study are listed in Table 1. LAT1 expression was determined by immunohistochemical staining with an affinity-purified rabbit polyclonal anti-human LAT1 antibody (1.2 mg/mL; 1:3200).[22] An oligopeptide corresponding to amino acid residues 497-507 of human LAT1 (CQKLMQVVPQET) was synthesized. The N-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanine. Antipeptide antibody was produced as described elsewhere.[23] For immunohistochemical analysis, antiserum was affinity purified as described previously.[23]

According to previous report,[3] immunohistochemical analysis of MUC1 expression was evaluated. First, staining density of MUC1 expression was classified into positive or negative, and, if positive, each tumor cell was further classified according to the expression pattern into polarized or depolarized expression. According to the percentage of tumor cells showing polarized MUC1 expression and that with depolarized MUC1 expression, MUC1 expression was classified into the high-grade polarized (HP), the low-grade polarized (LP), or the depolarized (DP) group. The classification of MUC1 expression status is as follows: (a) HP when positive percentage of tumor

Table 1. Technical Details of the Antibodies Employed in this Study

Antibody	Clone	Company, City (Country)	Dilution
MUC1 (Ma 552)	Rabbit monoclonal	Novocastra Laboratories Ltd, Newcastle (United Kingdom)	1:100
GLUT1 (AB15309)	Rabbit polyclonal	Abcam, Tokyo (Japan)	1:400
GLUT3	Rabbit polyclonal	Abcam, Tokyo (Japan)	1:200
Hexokinase I (AB55144)	Rabbit monoclonal	Abcam, Tokyo (Japan)	1:200
HIF-1α (NB100-123)	Mouse monoclonal	Novus Biologicals, Oakville, Ontario (Canada)	1:50
VEGF	Mouse monoclonal	Immuno-Biological Laboratories Co Ltd, Fujioka (Japan)	1:200
CD34	Mouse monoclonal	Nichirei, Tokyo (Japan)	1:800
p53 (D07)	Mouse monoclonal	DAKO, Tokyo (Japan)	1:50
EGFR	Mouse monoclonal	Novocastra Laboratories Ltd, Newcastle (United Kingdom)	1:100
PTEN	Rabbit monoclonal	Cell Signaling Technology, Inc, Danvers, MA (United States)	1:50
phosph-AKT	Rabbit polyclonal	Cell Signaling Technology, Inc, Danvers, MA (United States)	1:200
phosph-mTOR	Rabbit monoclonal	Cell Signaling Technology, Inc, Danvers, MA (United States)	1:80
phosph-S6K	Rabbit monoclonal	Cell Signaling Technology, Inc, Danvers, MA (United States)	1:100

Abbreviations: Glut1, glucose transporter 1; Glut3, glucose transporter 3; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analogue; mTOR, mammalian target of rapamycin; CD, cluster of differentiation.

cells with polarized MUC1 expression is more than 50% and positive percentage of tumor cells with depolarized MUC1 expression is less than 10%, (b) LP when positive percentage of tumor cells with polarized MUC1 expression is less than 50% and positive percentage of tumor cells with depolarized MUC1 expression is less than 10%, and (c) DP when positive percentage of tumor cells with depolarized MUC1 expression is more than 10% regardless of positive percentage with polarized MUC1 expression. According to the definition, the patient with tumor showing no MUC1 expression was classified into the LP group.

The expression of Glut1, Glut3, and EGFR was considered positive if distinct membrane staining was present. Five fields (×400) were analyzed to determine the frequency of the HIF-1α-stained nuclei and hexokinase I–stained cytoplasm. For Glut1, Glut3, EGFR, HIF-1α, and hexokinase I, a semiquantitative scoring method was used as follows: 1 = <10%, 2 = 10% to 25%, 3 = 25% to 50%, 4 = 51% to 75%, and 5 = >75% of cells positive. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive. The detailed protocol for immunostaining was published elsewhere.[18,21] LAT1 expression was considered positive only if distinct membrane staining was present. Staining intensity was scored as follows: 1 = ≤10% of tumor area stained, 2 = 11% to 25% stained, 3 = 26% to 50% stained, and 4 = ≥51% stained. The tumors in which stained tumor cells made up more than 10% of the tumor were graded as positive.

The expression of VEGF was quantitatively assessed according to the percentage of immunoreactive cells in the total of 1000 neoplastic cells. The number of CD34-positive vessels was counted in 4 selected hot spots in a ×400 field (0.26 mm^2 field area). MVD was defined as the mean count of microvessels per 0.26 mm^2 field area. For p53, microscopic examination for the nuclear reaction product was performed and scored. p53 expression in more than 10% of tumor cells was defined as high expression. p-AKT, p-mTOR, and p-S6K were considered positive if membranous and/or cytoplasmic staining was present, and PTEN was positive if nuclear staining. For p-AKT, p-mTOR, p-S6K, and PTEN, a semiquantitative scoring method was used: 1 = <10%, 2 = 10% to 25%, 3 = 25% to 50%, 4 = 51% to 75%, and 5 = >75% of cells positive. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive. Sections were assessed using light microscopy in a blinded fashion by at least 2 of the authors.

Statistical Analysis

Probability values <.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association of 2 categorical variables. The Kaplan–Meier method was used to estimate survival as a function of time, and survival difference were analyzed by the log-rank test. Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using JMP 8 (SAS Institute Inc., Cary, NC) for Windows.

Results

Immunohistochemical Analysis

Each protein revealed a profile pattern of the unique expression. The immunohistochemical staining of MUC1,

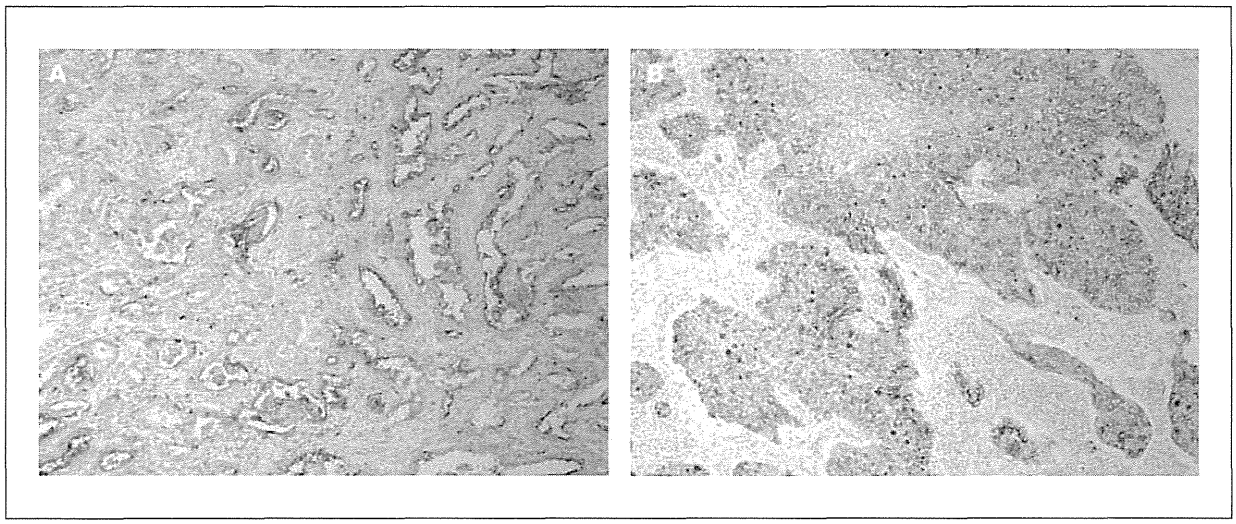

Figure 1. Immunohistochemical staining of MUC1 in pulmonary adenocarcinoma
A, High-grade polarized expression (HP) pattern of MUC1 in a well-differentiated adenocarcinoma. B, Depolarized expression (DP) pattern of MUC1 in a poorly differentiated adenocarcinoma.

Glut1, Glut3, hexokinase I, HIF-1α, VEGF, CD34, LAT1, EGFR, PTEN, p-Akt, p-mTOR, p-S6K, and p53 was evaluated for the 126 primary lesions. Figure 1 represents the immunohistochemical staining of MUC1 expression. Of all 126 patients, HP, LP, and DP group were 50 (39.7%), 35 (27.8%), and 41 (32.5%), respectively. Glut1 and Glut3 were detected in tumor cells and localized predominantly on their plasma membrane. A positive rate of Glut1 and Glut3 expression was recognized in 67.5% (85/126) and 11.1% (12/126), respectively. A positive expression of HIF-1α was predominantly recognized in the cytoplasm with some nuclear staining and was recognized in 87.3% (101/126). A positive expression of hexokinase I was recognized in the cytoplasm and/or membrane of neoplastic cells and was recognized in 68.3% (86/126). The median rate of VEGF positivity was 35% (range, 3% to 75%), and the value of 35% was chosen as a cutoff point. Positive expression was recognized in 42.9% (54/126). The median numbers of CD34 was 25 (2 to 65), and the value of 25% was chosen as a cutoff point. Positive expression was recognized in 48.4% (61/126). A positive expression of EGFR, PTEN, p-AKT, p-mTOR, p-S6K, and p53 was 55.6% (70/126), 15.1% (19/126), 49.2% (62/126), 60.0% (68/126), and 48.4% (61/126), respectively.

Demographics of Patients According to MUC1 Expression

The demographic result of the patients according to MUC1 expression is listed in Table 2. The frequency of male, non-AC, vascular invasion, lymphatic permeation, Glut1, HIF-1α, VEGF, MVD, LAT1, EGFR, and loss of PTEN was significantly higher in DP group than in HP group. Non-AC, Glut1, HIF-1α, VEGF, MVD, and LAT1 were significantly higher in DP group than in LP group. Compared with HP group, non-AC, the expression of Glut1, EGFR, loss of PTEN, and p-mTOR was significantly higher in LP group. As lung AC patients were distributed into 3 groups, the analysis of AC patients was further performed (Table 3). The frequency of advanced stage, lymphatic permeation, Glut1, CD34, LAT1, and EGFR was significantly higher in the DP group than in the HP group. MVD yielded a statistically significant difference between the LP and DP groups. Advanced stage and EGFR yielded a statistically significant difference between the HP and LP groups.

Next, we also performed a quantitative analysis of these molecular markers according to MUC1 expression (Figure 2). The mean scoring of Glut1, HIF-1α, LAT1, and EGFR, VEGF positivity, and number of microvessels (CD34), yielded a statistically significant difference among the 3 groups (HP vs LP, HP vs DP, and LP vs DP). All these variables showed a statistically significant difference between HP and DP. The mean scoring of Glut1, HIF-1α, and LAT1, VEGF positivity, and number of microvessels (CD34) showed a statistically significant difference between LP and DP. The mean scoring of Glut1 and EGFR showed a statistically significant difference between HP and LP.

MUC1 Status and Survival Analysis

For all patients, 5-year survival rates of the HP, LP, and DP patients were 79.4%, 61.5%, and 43.9%, respectively. A statistically significant difference in the overall survival

Table 2. Different Variables According to MUC1 Expression in All Patients

Different Variables		Total (n= 126)	HP (n = 50)	LP (n = 35)	DP (n = 41)	P		
						HP/LP	HP/DP	LP/DP
Age (Years)	≤65/>65	56/70	22/28	16/19	18/23	1.000	1.000	1.000
Gender	Male/female	81/45	26/24	22/13	33/8	.377	**.007**	.122
Histology	AC/non-AC	82/44	47/3	23/12	12/29	**.001**	**<.001**	**.002**
p stage	I + II/III	88/38	38/12	24/11	26/15	.467	.249	.808
PI	Positive/negative	34/92	13/37	7/28	14/27	.608	.490	.204
Vas	Positive/negative	59/67	17/33	16/19	26/15	.366	**.006**	.165
Ly	Positive/negative	58/68	16/34	16/19	26/15	.256	**.003**	.165
LN meta	Positive/negative	50/76	23/27	16/19	11/30	1.000	.081	.098
Glut1	Positive/negative	85/41	21/29	24/11	40/1	**.026**	**<.001**	**<.001**
Glut3	Positive/negative	14/112	5/45	2/33	7/34	.694	.364	.165
Hexo I	Positive/negative	86/40	32/18	21/14	33/8	.821	.104	.075
HIF-1α	Positive/negative	101/25	35/15	27/8	39/2	.620	**.002**	**.037**
VEGF	Positive/negative	54/72	16/34	11/24	27/14	1.000	**.001**	**.005**
CD34	Positive/negative	61/65	16/34	15/20	30/11	.363	**<.001**	**.010**
LAT1	Positive/negative	51/75	11/39	10/25	30/11	.610	**<.001**	**<.001**
EGFR	Positive/negative	70/56	16/34	23/12	31/10	**.003**	**<.001**	.447
PTEN	Positive/negative	19/107	14/36	2/33	3/38	**.011**	**.014**	1.000
p-Akt	Positive/negative	62/64	29/21	18/17	15/20	.658	.191	.632
p-mTOR	Positive/negative	68/58	32/18	13/22	23/18	**.016**	.520	.112
p-S6K	Positive/negative	86/40	34/16	20/15	32/9	.363	.348	.082
p53	Positive/negative	61/65	20/30	21/14	20/21	.081	.524	.363

NOTE: Boldfaced entries showing statistically significant difference.

Abbreviations: HP, high-grade polarized expression; LP, low-grade polarized expression; DP, depolarized expression; AC, adenocarcinoma; p stage, pathological stage; PI, pleural involvement;Vas, vascular invasion; Ly, lymphatic permeation; LN meta, lymph node metastasis; Glut1, glucose transporter 1; Glut3, glucose transporter 3; Hexo I, hexokinase I; HIF-1α, hypoxia inducible factor-1 alpha;VEGF, vascular endothelial growth factor; LAT1, l-type amino acid transporter 1; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; HP/LP, statistical comparison of HP and LP; HP/DP, statistical comparison of HP and DP; DP/LP, statistical comparison of DP and LP.

was observed between HP and LP patients ($P = .0394$), and between HP and DP patients ($P = .0002$), demonstrating no significant difference between LP and DP patients (Figure 3A). The 5-year survival rates of patients with HP or LP and those with DP were 72.0% and 43.9%, respectively ($P < .001$). The 5-year survival rates according to different variables are listed in Table 4. Univariate analysis demonstrated that male, non-AC, disease stage and the expression of MUC1, Glut1, HIF-1α, VEGF, CD34, LAT1, and EGFR were significantly associated with poor outcome. Multivariate analysis demonstrated that disease stage was an independent prognostic factor for predicting poor outcome.

For AC patients, 5-year survival rates of the HP, LP, and DP patients were 84.5%, 56.5%, and 33.3%, respectively. A statistically significant difference in the overall survival was recognized between HP and LP patients ($P = .0263$), between LP and DP patients ($P = .0454$), and between HP and DP patients ($P < .0001$). The 5-year survival rates of patients with HP or LP and those with DP were 76.7% and 33.3%, respectively ($P < .001$). The 5-year survival rates according to different variables are listed in Table 5. Univariate analysis demonstrated that disease stage and the expression of MUC1, Glut1, HIF-1α, VEGF, CD34, LAT1, and EGFR were significantly associated with poor outcome. By multivariate analysis, there was no statistically significant independent prognostic factor.

For non-AC patients, the 5-year survival rates of patients with HP or LP and those with DP were 50.0% and 44.8%, respectively, demonstrating no significant difference ($P = .8821$).

Discussion

This is a retrospective study to evaluate the relationship between MUC1 expression and various molecular markers in patients with resected NSCLC. Depolarized MUC1 expression was significantly related to poor outcome and was closely associated with glucose metabolism (Glut1), hypoxia (HIF-1α), angiogenesis (VEGF and MVD), amino acid metabolism (LAT1), and EGFR expression (Figure 4). High-grade polarized MUC1 expression was associated with favorable prognosis and AC. The expression

Table 3. Different Variables According to MUC1 Expression in Lung Adenocarcinoma

Different Variables		Total (n = 82)	HP (n = 47)	LP (n = 23)	DP (n = 12)	P		
						HP/LP	HP/DP	LP/DP
Age (Years)	≤65/>65	38/44	20/27	12/11	6/6	.610	.749	1.000
Gender	Male/female	43/39	23/24	11/12	9/3	1.000	.193	.162
p stage	I + II/III	54/28	37/10	12/11	5/7	**.029**	**.027**	.724
PI	Positive/negative	21/61	12/35	4/19	5/7	.552	.299	.220
Vas	Positive/negative	32/50	14/33	10/13	8/4	.291	.041	.289
Ly	Positive/negative	33/49	14/33	10/13	9/3	.291	**.007**	.151
LN meta	Positive/negative	36/46	21/26	12/11	3/9	.615	.326	.162
Glut1	Positive/negative	42/40	18/29	13/10	11/1	.201	**.001**	.055
Glut3	Positive/negative	7/75	4/43	1/22	2/10	1.000	.591	.265
Hexo I	Positive/negative	50/32	29/18	12/11	9/3	.606	.509	.281
HIF-1α	Positive/negative	60/22	32/15	17/6	11/1	.782	.380	.150
VEGF	Positive/negative	30/52	16/31	7/16	7/5	1.000	.153	.185
CD34	Positive/negative	33/49	14/33	9/14	10/2	.588	**.002**	**.016**
LAT1	Positive/negative	18/64	8/39	4/19	6/6	1.000	**.026**	.059
EGFR	Positive/negative	39/43	14/33	17/6	8/4	**<.001**	**.041**	.706
PTEN	Positive/negative	18/64	14/33	3/20	1/11	.149	.160	1.000
p-Akt	Positive/negative	52/30	27/20	17/6	8/4	.201	.744	.706
p-mTOR	Positive/negative	46/36	29/18	9/14	8/4	.124	1.000	.164
p-S6K	Positive/negative	58/24	33/14	15/8	10/2	.785	.482	.434
p53	Positive/negative	36/46	19/28	13/10	4/8	.307	.749	.289

NOTE: Boldfaced entries showing statistically significant difference.

Abbreviations: HP, high-grade polarized expression; LP, low-grade polarized expression; DP, depolarized expression; p stage, pathological stage; PI, pleural involvement; Vas, vascular invasion; Ly, lymphatic permeation; LN meta, lymph node metastasis; Glut1, glucose transporter 1; Glut3, glucose transporter 3; Hexo I, hexokinase I; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; LAT1, l-type amino acid transporter 1; EGFR, epidermal growth factor receptor; PTEN, Phosphatase and tensin analog; mTOR, mammalian target of rapamycin; HP/LP, statistical comparison of HP and LP; HP/DP, statistical comparison of HP and DP; DP/LP, statistical comparison of DP and LP.

of hypoxic markers, LAT1, and EGFR was significantly lower in HP patients than in DP patients. In AC patients, glucose metabolism, MVD, amino acid metabolism, and EGFR yielded a statistically significant difference between HP and DP patients. Our results suggest that glucose and amino acid metabolism, hypoxia, angiogenesis, and EGFR expression have a crucial role in the development of cancer progression and metastases in NSCLC patients with depolarized MUC1 expression. In the clinicopathological study using tumor specimens, to our knowledge, there is still no data about the relationship between MUC1 expression and these biomarkers in human neoplasms.

Recently, several reports have documented that MUC1 expression is correlated with tumor differentiation and postoperative survival in patients with NSCLC.[3,7-10] Nagai et al[3] described that depolarized MUC1 expression was a significant and independent prognostic factor to predict poor postoperative prognosis in patients with pulmonary AC and LP or DP expression was mostly observed in moderately to poorly differentiated AC patients. Situ et al[10] also reported that MUC1 has a prognostic relevance in early-stage lung AC. This is corresponding to the results of our study. However, 2 studies reported that MUC1 expression was correlated with worse prognosis not AC but SQC, demonstrating a contradictory result.[5,7] The other reports demonstrated that MUC1 status alone was not correlated to survival in lung cancer.[24,25] Therefore, Nagai et al[3] conducted a more detailed MUC1 status classification (HP, LP, and DP) and showed a significant difference in postoperative survival between HP and DP. Although the expression profile of MUC1 is different among the studies and the methods used in the studies also have a different technique, we selected the expression analysis of MUC1 according to the study by Nagai et al.[3] To assume prospective validation, these molecular techniques will need optimization and standardization in further trials.

In the present study, the expression profile of molecular markers was markedly different between HP and DP groups. Kikami et al[11] suggest that hypoxia enhances MUC1 expression in lung AC cell line. As this is an in vitro study, it remains unknown whether hypoxia strongly enhanced depolarized MUC1 expression as compared with polarized MUC expression. However, our results suggest that the degree of hypoxia was higher in the tumors with depolarized MUC1 expression than in those with

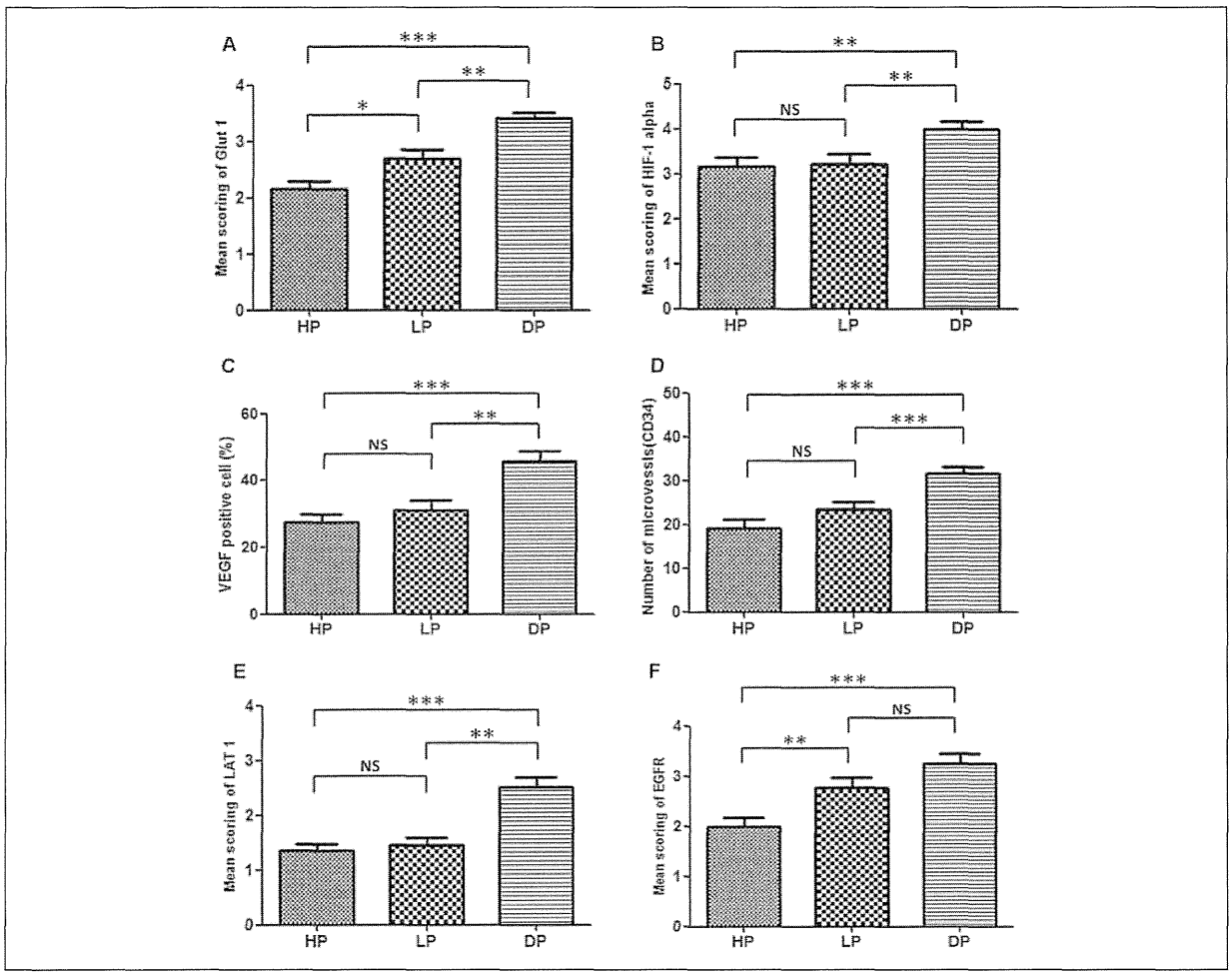

Figure 2. Quantitative analysis of mean scoring of Glut1, HIF-1α, LAT1 and EGFR, VEGF positivity, and number of microvessels (CD34) according to MUC1 expression

A, Mean scoring of Glut1 showing statistically significant difference between high-grade polarized expression (HP) and low-grade polarized expression (LP), between LP and depolarized expression (DP), and between HP and DP. B, Mean scoring of HIF-1α showing statistically significant difference between HP and DP, and between LP and DP, but not between HP and LP. C, VEGF positivity showing statistically significant difference between HP and DP, and between LP and DP, but not between HP and LP. D, Number of microvessels (CD34) showing statistically significant difference between HP and DP, and between LP and DP, but not between HP and LP. E, Mean scoring of LAT1 showing statistically significant difference between HP and DP, and between LP and DP, but not between HP and LP. F, Mean scoring of EGFR showing statistically significant difference between HP and LP, and between HP and DP, but not between LP and DP. *P* values indicate significance and were calculated using Fisher's exact test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, NS indicates nonsignificance.

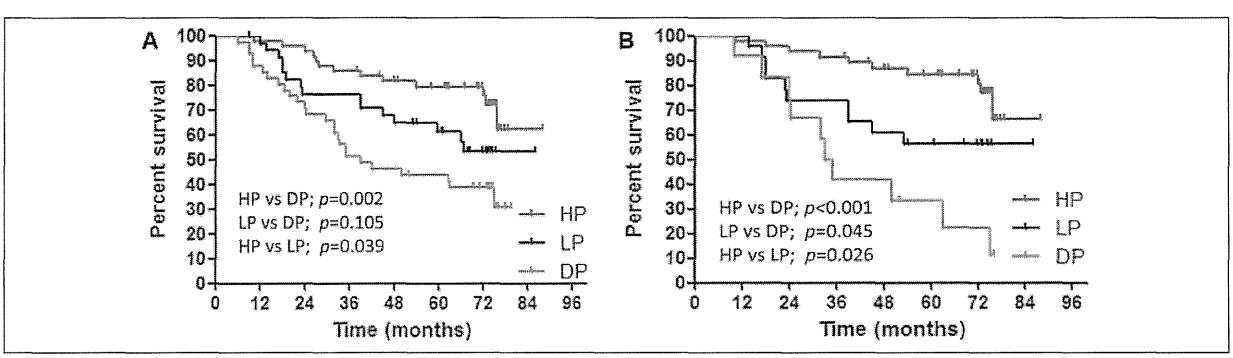

Figure 3. High-grade polarized expression (HP), low-grade polarized expression (LP), and depolarized expression (DP)

A, Postoperative survival of non–small cell lung cancer patients according to MUC1 expression pattern. B, Postoperative survival of pulmonary adenocarcinoma patients according to MUC1 expression pattern.

Table 4. Univariate and Multivariate Survival Analysis in All Patients

Different Variables		Univariate Analysis 5-Year Survival Rate (%)	Univariate Analysis *P*	Multivariate Analysis *P*
Age (years)	≤65/>65	57.6/67.1	.581	
Gender	Male/ female	55.7/75.4	**.036**	.484
Histology	AC/ non-AC	70.2/48.9	**.011**	.328
p stage	I + II/III	71.4/40.9	**<.001**	**.007**
MUC1	HP/LP and DP	72.0/43.9	**<.001**	.839
Glut1	Positive/ negative	49.5/89.9	**<.001**	**.004**
Glut3	Positive/ negative	59.6/62.0	.558	
Hexo I	Positive/ negative	60.7/67.3	.474	
HIF-1α	Positive/ negative	56.4/88.0	**.011**	.379
VEGF	Positive/ negative	52.5/70.8	**<.001**	.956
CD34	Positive/ negative	48.5/76.2	**<.001**	.554
LAT1	Positive/ negative	57.6/74.1	**.038**	.618
EGFR	Positive/ negative	49.1/79.9	**.005**	.235
PTEN	Positive/ negative	82.6/59.4	.076	
p-Akt	Positive/ negative	62.5/63.2	.818	
p-mTOR	Positive/ negative	66.5/58.6	.542	
p-S6K	Positive/ negative	52.8/69.9	.098	
p53	Positive/ negative	61.7/63.7	.953	

NOTE: Boldfaced entries showing statistically significant difference.
Abbreviations: HP, high-grade polarized expression; LP, low-grade polarized expression; DP, depolarized expression; AC, adenocarcinoma; p stage, pathological stage; PI, pleural involvement; Vas, vascular invasion; Ly, lymphatic permeation; LN meta, lymph node metastasis; Glut1, glucose transporter 1; Glut3, glucose transporter 3; Hexo I, hexokinase I; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; LAT1, l-type amino acid transporter 1; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; HP/LP, statistical comparison of HP and LP; HP/DP, statistical comparison of HP and DP; DP/LP, statistical comparison of DP and LP.

Table 5. Univariate and Multivariate Survival Analysis in Adenocarcinoma

Different Variables		Univariate Analysis 5-Year Survival Rate (%)	Univariate Analysis *P*	Multivariate Analysis *P*
Age (years)	≤65/>65 years	64.6/75.7	.586	
Gender	Male/ female	64.4/76.7	.158	
p stage	I + II/III	80.0/48.2	**.002**	.524
MUC1	HP/LP and DP	76.7/33.3	**<.001**	.269
Glut1	Positive/ negative	51.4/89.6	**<.001**	**.021**
Glut3	Positive/ negative	64.2/68.7	.972	
Hexo I	Positive/ negative	67.0/75.0	.253	
HIF-1α	Positive/ negative	62.4/90.9	**.017**	.821
VEGF	Positive/ negative	52.7/80.3	**<.001**	.931
CD34	Positive/ negative	43.5/87.7	**<.001**	.089
LAT1	Positive/ negative	62.9/87.5	**.012**	.380
EGFR	Positive/ negative	55.1/83.6	**.016**	.413
PTEN	Positive/ negative	82.6/66.6	.220	
p-Akt	Positive/ negative	66.8/76.2	.125	
p-mTOR	Positive/ negative	67.9/72.9	.400	
p-S6K	Positive/ negative	52.1/75.9	.152	
p53	Positive/ negative	63.0 / 75.6	.204	

NOTE: Boldfaced entries showing statistically significant difference.
Abbreviations: HP, high-grade polarized expression; LP, low-grade polarized expression; DP, depolarized expression; p stage, pathological stage; PI, pleural involvement; Vas, vascular invasion; Ly, lymphatic permeation; LN meta, lymph node metastasis; Glut1, glucose transporter 1; Glut3, glucose transporter 3; Hexo I, hexokinase I; HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; LAT1, l-type amino acid transporter 1; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin analog; mTOR, mammalian target of rapamycin; HP/LP, statistical comparison of HP and LP; HP/DP, statistical comparison of HP and DP; DP/LP, statistical comparison of DP and LP.

polarized MUC1 expression, and HIF-1α is upregulated by hypoxia and induces Glut1 expression and angiogenesis. Moreover, the activation of EGFR pathway and amino acid transporter (LAT1) also may play a crucial role in the tumor progression and metastases. This is also supported by the findings that MUC1 expression was found in highly vascularized tumors, a significant coexpression with multiple angiogenic factors and their receptors.[9] Moreover,

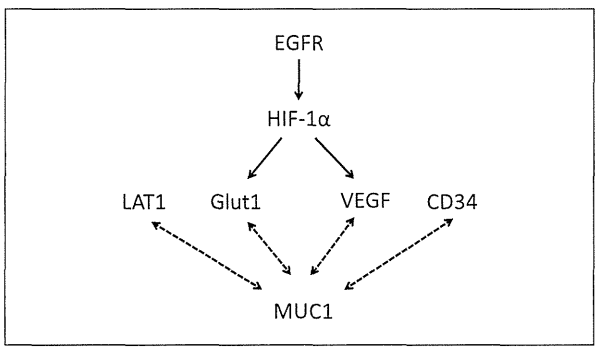

Figure 4. MUC1 expression is significantly associated with the expression of glucose metabolism (Glut1), hypoxia (HIF-1α), angiogenesis (VEGF and CD34), amino acid metabolism (LAT1), and EGFR. HIF-1α is a downstream component of EGFR pathway, and the expression of Glut1 and VEGF is regulated by hypoxia (HIF-1α) in a HIF-1-dependent way

one experimental study demonstrated that suppression of MUC1 synthesis downregulates expression of EGFR at both the mRNA and protein level in human carcinoma cells.[26] However, our study indicated that the prognostic role of MUC1 expression seemed to be weak as compared with that of Glut1 expression. As the sample size is small, further investigation is warranted.

Nowadays, the development of immunotherapy is important as new option for cancer therapy. MUC1 core protein may be a useful target molecule for immunotherapy in breast cancer, lung cancer, and other malignancies expressing MUC1.[27,28] Most patients with NSCLC had a positive expression of MUC1, therefore a MUC1-targeted immunotherapy may be appropriate for NSCLC showing MUC1 expression. Especially, the MUC1-targeted therapy will be expected to be administered for NSCLC with depolarized MUC1 expression showing poor outcome. Moreover, MUC1 expression is correlated with hypoxia and Glut1 expression. 2-^{18}F-FDG PET has been investigated for monitoring tumor response to chemotherapy, radiotherapy, and molecular targeted therapy.[29,30] The amount of ^{18}F-FDG uptake in human neoplasm is determined by the presence of glucose metabolism (Glut1), hypoxia (HIF-1α), and angiogenesis (VEGF and MVD).[20] Thus, ^{18}F-FDG PET may be useful for monitoring the response of NSCLC treated by MUC1-targeted therapy. Moreover, ^{18}F-FDG PET may be effective for differentiating between polarized MUC1 and depolarized MUC1 expression tumors.

In conclusion, depolarized MUC1 expression was significantly associated with poor outcome in NSCLC, especially in lung AC. Glucose metabolism (Glut1), hypoxia (HIF-1α), angiogenesis (VEGF and MVD), amino acid metabolism (LAT1), and EGFR expression have a crucial role in the development of depolarized MUC1 expression. A novel classification of MUC1 expression pattern (HP, LP, and DP) may be useful for predicting postoperative outcome in NSCLC, especially in pulmonary AC.

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Declaration of Conflicting Interests

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ORIGINAL PAPER

Thymidylate synthase expression is closely associated with outcome in patients with pulmonary adenocarcinoma

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Abstract The aim of this study is to elucidate the prognostic significance of thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) in completely resected non-small cell lung cancer (NSCLC). One hundred and sixty patients with NSCLC were included in this study. Tumor sections were stained by immunohistochemistry for TS, OPRT, DPD, glucose transporter 1 (Glut1), hypoxia inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), microvessel density (MVD) determinated by CD34, epidermal growth factor receptor (EGFR), phosph-Akt, phosph-mammalian target of rapamycin (mTOR) and p53. TS, OPRT and DPD were positively expressed in 46, 71 and 54%, respectively. The expression of TS and OPRT was significantly higher in patients with non-adenocarcinoma (non-AC) ($n = 53$) than adenocarcinoma (AC) ($n = 107$), and DPD expression was higher in adenocarcinoma as compared with non-adenocarcinoma. A positive TS expression was an independent prognostic factor for predicting a poor outcome in patients with AC, but not in those with non-AC. In AC patients, TS expression was significantly associated with advanced stage, lymph node metastases, vascular invasion, Glut1, HIF-1α, angiogenesis, EGFR signaling pathway and p53. In patients with non-AC, TS expression was not closely correlated with outcome and these biomarkers. A positive TS expression was a powerful prognostic factor to predict a poor outcome in completely resected AC patients.

Keywords TS · OPRT · DPD · NSCLC · Adenocarcinoma · Prognosis

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Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death and has a dismal prognosis. To improve the outcome of NSCLC patients, the biomarkers that may predict the prognosis and response to the specific therapy should be established. Tumor staging and performance status have been consistently shown to be the most powerful prognostic tool for predicting the outcome of NSCLC patients [1]. However, there has been no established clinical marker, which correlates with the response to the treatment and the prognosis in patients with NSCLC.

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) [2]. TS is also a target enzyme of 5-fluorouracil (5-FU), which is an anticancer chemotherapeutic agent for various human cancers [3]. The anticancer activity of 5-FU has been described to

be closely associated with the intratumoral expression of TS, orotate phosphoribosyltransferase (OPRT) and dihydropyrimidine dehydrogenase (DPD) [4]. TS expression has been described to be significantly correlated with proliferative activity and poor prognosis in patients with NSCLC [5]. Several researchers had documented that TS expression in patients with NSCLC is significantly higher in squamous cell carcinoma (SQC) compared with adenocarcinoma (AC), and a positive correlation is observed between TS expression and disease stage, lymph node metastasis or tumor differentiation [6, 7]. However, it is unclear whether the prognostic significance of TS expression is different according to the histology of NSCLC. Recently, TS expression had been described to be correlated with the hypoxic markers such as hypoxia inducible factor-1α (HIF-1α), glucose transporter 1 (Glut1) and vascular endothelial growth factor (VEGF) in colorectal cancer [8]. Moreover, experimental study using NSCLC cell lines had documented that the combination of oral fluorouracil S-1 and epidermal growth factor receptor (EGFR) inhibitor gefitinib showed a synergistic antitumor effect, and gefitinib induced the down-regulation of TS [9]. These reports suggest that TS expression is related to EGFR signaling pathway and hypoxic condition in cancers. However, it remains unknown whether TS expression is associated with hypoxia-related protein or EGFR signaling pathway in human neoplasm.

Nakano et al. [4] reported the prognostic role of TS, OPRT and DPD for NSCLC patients postoperatively treated by a combination of tegafur and uracil (UFT). However, it remains unknown whether OPRT and DPD expression has a prognostic role in NSCLC patients without 5-FU adjuvant therapy.

To elucidate the prognostic role of TS, OPRT and DPD, we conducted an immunohistochemical examination of these biomarkers in patients with resected NSCLC. In addition, the relationship between TS expression, and hypoxia-related protein [HIF-1α, Glut1, VEGF and microvessel density (MVD) determined by CD34], EGFR signaling pathway [EGFR, phosph-Akt and phosph-mammalian target of rapamycin (mTOR)] and cell cycle regulator (p53) were also presented.

Materials and methods

Patients

Between October 2002 and September 2004, we analyzed 173 consecutive patients with NSCLC who underwent resection by either lobectomy or pneumonectomy with mediastinal lymph node dissection at Shizuoka Cancer Center. Of these patients, 13 patients were excluded for further studies because the tissue specimens were not available. Thus, a total of 160 patients (97 men, 63 women) were eligible in the study. The study protocol was approved by the institutional review board.

The age of the patients ranged from 43 to 83 years, and the median age at the time of surgery was 67 years. The tumor specimens were histologically classified according to the criteria of the World Health Organization. Pathologic tumor-node-metastasis (TNM) stages were established using the International System for Staging Lung Cancer adopted by the American Joint Committee on Cancer and the Union Internationale Centre le Cancer [10]. Histologically, 107 patients had adenocarcinoma (AC), 48 had squamous cell carcinoma (SQC), and five had large cell carcinoma (LCC). Of the total patients, 106, 25 and 29 had stage I, II and III diseases, respectively. The patients who underwent lobectomy and pneumonectomy were 104 and two patients in stage I, respectively, 23 and two patients in stage II, respectively, and 24 and five patients in stage III, respectively. As postoperative adjuvant therapies, platinum-based chemotherapy, radiation and oral administration of tegafur (a fluorouracil derivative drug) were carried out in 2, 1 and 6 patients, respectively. The two patients who received platinum-based chemotherapy (carboplatin plus paclitaxel) had stage III, the one patient treated by radiation had stage III, and the six patients receiving oral administration of tegafur had stage I. Intraoperative therapy was not performed on any patient. The postoperative clinical course was assessed by analyzing outpatient medical records and by marking telephone inquiries. The day of surgery was considered the starting day for counting postoperative survival. The follow-up duration ranged from 7 to 102 months (median, 73 months).

Immunohistochemical staining

Immunohistochemical staining was performed according to the procedure described in the previous reports [4, 11]. The following antibodies were used: a rabbit polyclonal antibody against TS (clone RTSSA; Taiho Pharmaceutical, Saitama, Japan; 1:1,600 dilution); a rabbit polyclonal antibody against OPRT (Taiho Pharmaceutical, Saitama, Japan; 1:1,200 dilution); a rabbit polyclonal antibody against DPD (clone RDPDPA; Taiho Pharmaceutical, Saitama, Japan; 1:500 dilution); a rabbit polyclonal against GLUT1 (AB15309, Abcam, Tokyo, Japan, 1:400 dilution); a mouse monoclonal against HIF-1α (NB100-123, Novus Biologicals, Inc., Littleton, 1:50 dilution); a monoclonal antibody against VEGF (Immuno-Biological Laboratories Co.,Ltd., Japan, 1:100 dilution); a mouse monoclonal antibody against CD34 (Nichirei, Tokyo, Japan, 1:800 dilution); a mouse monoclonal antibody against EGFR (Novovastra laboratories Ltd., Newcastle, UK, 1:100

dilution); a rabbit polyclonal antibody against phosph-Akt (Abcam, Tokyo, Japan, 1:200 dilution); a rabbit monoclonal antibody against phosph-mTOR (Cell signaling, 80 dilution); a mouse monoclonal antibody against p53 (D07; DAKO, 1:50 dilution). Antibodies against TS, OPRT and DPD were kindly donated by Taiho (Tokyo, Japan). The human colon cancer cell line DLD-1/FrUrd was used as a positive control for the staining of TS. Sections of resected lung tumors to express OPRT were used as positive controls for the staining of OPRT. The human pancreatic cancer cell line MIAPaCa-2 was used as a positive control for the staining of DPD. For negative control, incubation step with the primary antibody was omitted.

The expression of TS, OPRT and DPD was considered if nuclei or cytoplasm staining was present. For TS, OPRT and DPD, a semiquantitative scoring method was used: 1 = <10%, 2 = 10–24%, 3 = 25–50%, 4 = 51–75% and 5 = >75% of cells positive. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive.

The expression of Glut1 and EGFR was considered positive if distinct membrane staining was present. Five fields (X400) were analyzed to determine the frequency of the HIF-1α-stained nuclei. p-Akt and p-mTOR were considered positive if membranous and/or cytoplasmic staining was present. For Glut1, HIF-1α,EGFR, p-Akt and p-mTOR, a semiquantitative scoring method was used: 1 = <10%, 2 = 10–24%, 3 = 25–50%, 4 = 51–75% and 5 = >75% of cells positive. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive.

The expression of VEGF was quantitatively assessed according to the percentage of immunoreactive cells in a total of 1,000 neoplastic cells. The number of CD34-positive vessels was counted in four selected hot spots in a X 400 field (0.26 mm^2 field area). MVD was defined as the mean count of microvessels per 0.26 mm^2 field area.

For p53, microscopic examination for the nuclear reaction product was performed and scored. According to previous report, p53 expression in more than 10% of tumor cells was defined as high expression. Sections were assessed using a light microscopic in a blinded fashion by at least two of the authors. To test interobserver variability, each section was reassessed by the same investigators after completion of the first assessment. The time interval between the first and second assessments was at least 4 weeks. Intraobserver variability was also determined by comparing the values of the first measurements of two investigators.

Statistical analysis

Probability values of <0.05 indicated a statistically significant difference. Data are presented as mean ± SD. Fisher's exact test was used to examine the association of two categorical variables. Correlation of different variables was analyzed using the nonparametric Spearman's rank test. The Kaplan–Meier method was used to estimate survival as a function of time, and survival difference was analyzed by the log-rank test. Overall survival (OS) was defined as the time between diagnosis and death from any cause. Progression-free survival (PFS) was defined as the time between diagnosis and the first recurrence of the disease (local–regional or distant recurrence). Multivariate analyses were performed using stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Immunohistochemical analysis

Each protein revealed a profile pattern of the unique expression. The immunohistochemical staining of TS, OPRT, DPD, Glut1, HIF-1α, VEGF, CD34, EGFR, p-Akt, p-mTOR and p53 was evaluated for the 160 primary lesions. Figure 1 represents the immunohistochemical staining of TS, OPRT and DPD.

TS, OPRT and DPD were positively expressed in 46% (74/160), 71% (114/160) and 54% (87/160), respectively. A positive rate of TS, OPRT and DPD was recognized in 34% (36/107), 61% (65/107) and 70% (75/107), respectively, of AC and 72% (38/53), 92% (49/53) and 23% (12/53), respectively, of non-AC. The mean scoring of TS, OPRT and DPD was 2.24 ± 0.88, 3.00 ± 1.2 and 2.54 ± 1.05, respectively, in all cases, 1.98 ± 0.88, 2.63 ± 1.15 and 2.86 ± 0.97, respectively, in AC and 2.79 ± 0.61, 3.77 ± 0.94 and 1.88 ± 0.89, respectively, in non-AC. The mean scoring of TS and OPRT expression was significantly higher in non-AC than AC ($P < 0.0001$) but that of DPD expression was significantly lower in non-AC than AC ($P < 0.0001$).

Glut1 was detected in tumor cells and localized predominantly on their plasma membrane. A positive rate of Glut1 expression was recognized in 56%. A positive expression of HIF-1α was predominantly expressed in the cytoplasm with some nuclear staining and was recognized in 76%. The staining pattern of VEGF was uniformly localized in the cytoplasm and/or membrane of neoplastic. The median rate of VEGF positivity was 33% (range, 2–86%), and the value of 33% was chosen as a cutoff point. Positive expression was recognized in 50%. The median number of CD34 was 25 (range, 3–53), and the value of cutoff point was 25. Positive expression of CD34 was seen in 49%. A positive expression of EGFR, p-Akt, and

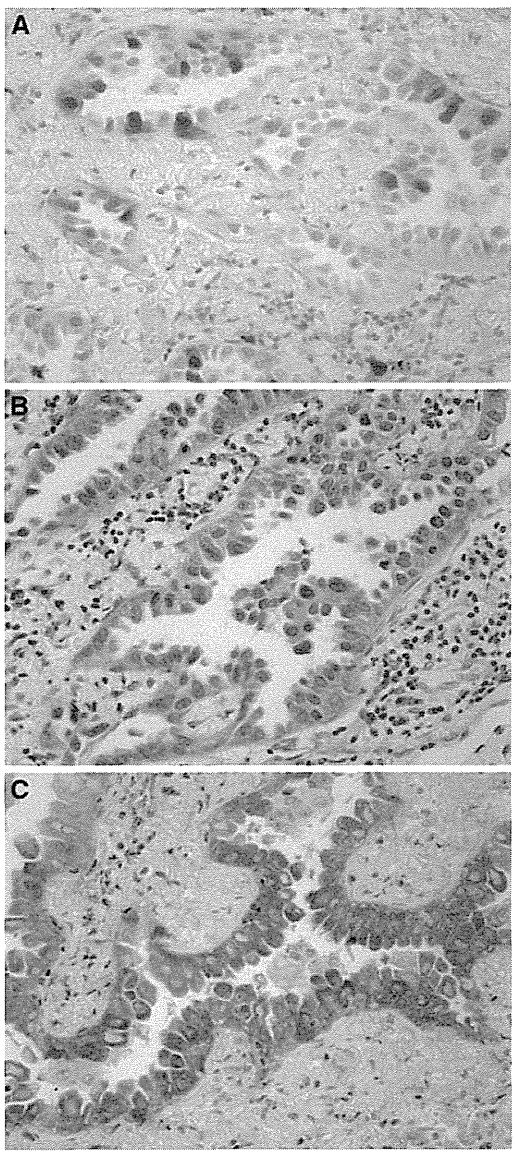

Fig. 1 Immunohistochemical staining of TS, OPRT and DPD in NSCLC. **a** TS is stained in nuclei (score 3). **b** OPRT is stained in mainly cytoplasm (score 4). **c** DPD is stained in nuclei (score 4)

p-mTOR was 56, 48 and 60%, respectively. High expression of p53 was recognized in 46%.

Demographics of patients according to TS, OPRT and DPD

The demographic result of the patients according to these biomarkers is listed in Table 1. In the analysis according to TS expression, histology, lymph node metastases, lymphatic permeation, OPRT, Glut1, HIF-1α, VEGF, CD34, EGFR and p53 yielded a statistically significant difference. In OPRT expression, histology, lymph node metastases, lymphatic permeation, vascular invasion, TS, Glut1, HIF-1α, VEGF, CD34, EGFR, p-mTOR and p53 yielded a statistically significant difference. In DPD expression, stage, histology, Glut1, CD34 and p-Akt yielded a statistically significant difference.

Using Spearman rank correlation, TS expression was significantly correlated with OPRT ($\gamma = 0.5374$, $P < 0.0001$), Glut1 ($\gamma = 0.6260$, $P < 0.0001$), HIF-1α ($\gamma = 0.5366$, $P < 0.0001$), VEGF ($\gamma = 0.6369$, $P < 0.0001$), CD34 ($\gamma = 0.5516$, $P < 0.0001$), EGFR ($\gamma = 0.3854$, $P < 0.0001$) and p-mTOR ($\gamma = 0.2783$, $P = 0.0004$) in all patients. In AC patients, TS expression was significantly correlated with OPRT ($\gamma = 0.5073$, $P < 0.0001$), DPD ($\gamma = 0.2511$, $P = 0.0088$), Glut1 ($\gamma = 0.6577$, $P < 0.0001$), HIF-1α ($\gamma = 0.5320$, $P < 0.0001$), VEGF ($\gamma = 0.6586$, $P < 0.0001$), CD34 ($\gamma = 0.5382$, $P < 0.0001$), EGFR ($\gamma = 0.3211$, $P = 0.0007$), p-Akt ($\gamma = 0.2689$, $P = 0.0049$) and p-mTOR ($\gamma = 0.2307$, $P = 0.0163$). In non-AC patients, however, TS expression was not significantly correlated with all of these biomarkers.

In the analysis according to the tumor differentiation of AC, no statistically significant difference in the mean scoring of TS expression was observed between well differentiated (1.85 ± 0.15) and moderately or poorly differentiated AC (2.06 ± 0.10) ($P = 0.2344$).

Survival analysis according to TS, OPRT and DPD

The 5-year survival rate for OS and PFS was 77.5 and 65.4%, respectively, and the median survival time was not reached. In the analysis of OS and PFS, a statistically significant poorer prognosis was observed in NSCLC ($n = 160$) with the positive expression of TS and DPD. In the analysis according to histology, a statistically significant difference in the OS and PFS was observed in AC patients ($n = 107$) with the positive expression of TS and DPD, but not OPRT. In patients with non-AC ($n = 53$), however, no statistically significant difference was observed according to the expression of TS, OPRT and DPD (Table 2). Figure 2 shows the Kaplan–Meier survival curves in patients with positive and negative for TS expression.

Univariate and multivariate analyses in all patients

Univariate and multivariate analyses of factors which affected the OS and PFS were performed. Univariate analysis demonstrated that a statistically significant difference in the OS was observed in gender, disease stage, TS, DPD, Glut1, HIF-1α, CD34 and EGFR. Multivariate analysis revealed that disease stage was an independent and significant factor to predict a poor prognosis (Table 3). By

Table 1 Different variables according to TS, OPRT and DPD

Variables	TS			OPRT			DPD		
	Positive ($n = 74$)	Negative ($n = 86$)	P value	Positive ($n = 114$)	Negative ($n = 46$)	P value	Positive ($n = 88$)	Negative ($n = 72$)	P value
Age									
≤65/> 65 yrs	35/39	36/50	0.525	48/66	23/23	0.384	38/50	33/39	0.751
Gender									
Male/female	51/23	46/40	0.052	73/41	24/22	0.211	49/39	48/24	0.194
Stage									
I + II/III	59/15	73/13	0.411	92/22	40/6	0.491	65/23	67/5	**0.002**
Histology									
AC/non-AC	36/38	71/15	**<0.001**	65/49	42/4	**<0.001**	76/12	31/41	**<0.001**
Lymph node metastasis									
Positive/negative	27/47	16/70	**0.012**	39/75	4/42	**<0.001**	26/62	17/55	0.4745
Pleural involvement									
Positive/negative	18/56	22/64	1.000	31/83	9/37	0.420	21/67	19/53	0.718
Vascular invasion									
Positive/negative	33/41	28/58	0.142	54/60	7/39	**<0.001**	34/54	27/45	1.000
Lymphatic permeation									
Positive/negative	37/37	24/62	**0.005**	53/61	8/38	**<0.001**	34/54	27/45	1.000
TS									
Positive/negative	(−)	(−)	(−)	68/46	6/40	**<0.001**	36/52	38/34	0.153
OPRT									
Positive/negative	68/6	46/40	**<0.001**	(−)	(−)	(−)	66/22	48/24	0.293
DPD									
Positive/negative	36/38	52/34	0.153	66/48	22/24	0.293	(−)	(−)	(−)
Glut1									
Positive/negative	62/12	27/59	**<0.001**	79/35	10/36	**<0.001**	38/50	51/21	**<0.001**
HIF-1$^{\alpha}$									
Positive/negative	70/4	52/34	**<0.001**	103/11	19/27	**<0.001**	68/20	54/18	0.852
VEGF									
Positive/negative	56/18	24/62	**<0.001**	75/39	5/41	**<0.001**	40/48	40/32	0.266
CD34									
Positive/negative	56/18	23/63	**<0.001**	70/44	19/27	**0.023**	39/49	50/22	**0.002**
EGFR									
Positive/negative	52/24	37/49	**0.001**	76/38	13/33	**<0.001**	49/39	40/32	1.000
p-Akt									
Positive/negative	36/38	41/45	1.000	58/56	19/27	0.298	57/31	20/52	**<0.001**
p-mTOR									
Positive/negative	48/26	48/36	0.332	79/35	17/29	**<0.001**	53/35	43/29	1.000
P53									
Positive/negative	47/27	26/60	**<0.001**	66/48	7/39	**<0.001**	36/52	37/35	0.205

TS thymidylate synthase, *OPRT* orotate phosphoribosyltransferase, *DPD* dihydropyrimidine dehydrogenase, *AC* adenocarcinoma, *Glut1* glucose transporter 1, *HIF-1α* hypoxia inducible factor-1α, *VEGF* vascular endothelial growth factor, *EGFR* epidermal growth factor receptor, *p-mTOR* phosph-mammalian target of rapamycin

Bold denotes statistically significance value

univariate analysis, a statistically significant difference in the PFS was observed in disease stage, TS, DPD, Glut1, HIF-1α, VEGF, CD34, EGFR and p-Akt. Multivariate analysis demonstrated that disease stage and a positive TS expression were independent prognostic factors to predict a poor outcome (Table 4).

Table 2 Survival analysis according to TS, OPRT and DPD

	TS			OPRT			DPD		
	Positive (%)	Negative (%)	*P* value	Positive (%)	Negative (%)	*P* value	Positive (%)	Negative (%)	*P* value
Total ($n = 160$)									
OS 5-yr rate (%)	67.5	86.1	**0.0002**	71.6	84.7	0.1536	71.9	91.3	**0.0022**
PFS 5-yr rate (%)	48.6	79.1	**<0.0001**	57.9	73.6	0.1409	56.1	86.9	**0.0005**
AC ($n = 107$)									
OS 5-yr rate (%)	61.1	90.1	**<0.0001**	74.6	93.7	0.0572	73.4	90.7	**0.0096**
PFS 5-yr rate (%)	30.5	83.1	**<0.0001**	60.0	78.1	0.2134	51.5	86.1	**0.0008**
Non-AC ($n = 53$)									
OS 5-yr rate (%)	73.6	66.7	0.5561	53.8	77.5	0.1962	70.0	71.7	0.2165
PFS 5-yr rate (%)	65.8	53.3	0.5532	46.2	70.0	0.1371	62.0	62.2	0.2022

TS thymidylate synthase, *OPRT* orotate phosphoribosyltransferase, *DPD* dihydropyrimidine dehydrogenase, *AC* adenocarcinoma, *Non-AC* non-adenocarcinoma, *OS* overall survival, *PFS* progression-free survival, 5-year rate, 5-year survival rate

Bold denotes statistically significance value

Univariate and multivariate analyses according to different variables

In the analysis of patients ($n = 131$) with early stage (stage I or II), the positive expression of TS and DPD was an independent prognostic factor for predicting a poor outcome in the OS and PFS. In patients with stage III ($n = 29$), there was no independent prognostic factors for predicting outcome. Next, we examined the survival analysis according to histological type. In AC patients ($n = 107$), disease staging and TS expression were independent and significant factors to predict a poor prognosis in the OS and PFS. In non-AC patients ($n = 53$), however, TS expression was not significantly associated with poor outcome in the OS and PFS.

Moreover, we compared the different variables between positive and negative TS expression in patients with AC. The positive expression of TS in AC patients was significantly associated with advanced stage, lymph node metastases, vascular invasion, glucose metabolism, hypoxia, angiogenesis, EGFR expression and cell cycle regulator.

Discussion

This is the retrospective study to evaluate the prognostic significance of TS, OPRT and DPD in patients with NSCLC. A multivariate analysis demonstrated the positive TS expression to be independently associated with an increased risk for poor OS and DFS in patients with AC, but not in those with non-AC. TS expression may be a useful marker for predicting postoperative outcome in AC patients. Limited to the analysis of AC patients, TS expression was significantly associated with OPRT, DPD, advanced stage, lymph node metastases, vascular invasion, glucose metabolism (Glut1), hypoxia (HIF-1α), angiogenesis (VEGF and CD34), EGFR/Akt/mTOR pathway and cell cycle regulator (p53). In patients with non-AC (almost SQC), TS expression seemed to be not correlated with outcome and these biomarkers.

High-level TS expression is related to an aggressive tumor phenotype and a poor outcome in a variety of malignant tumors [12, 13]. TS levels are generally lower in adenocarcinoma and in some large cell carcinoma than in squamous cell carcinoma [14]. In lung cancer, TS expression has been described to be higher in neuroendocrine carcinoma of the lung than in squamous cell carcinoma [15]. In our series, TS expression was also significantly higher in non-AC (almost SQC) than in AC. Ceppi et al. [6] had documented higher expression of TS in pulmonary SQC compared with non-squamous histotypes and described that a strong correlation was observed between *TS* mRNA and protein levels. But they had no description about the relationship between TS expression and prognosis in patients with pulmonary SQC. There was a significantly higher proliferative activity in TS-high tumor in NSCLC patients [5]. When stratified according to histology, there was a significant difference in AC, but no difference in SQC. In our study, TS expression in AC patients was closely associated with hypoxia-related protein expression and tumor aggressiveness. Recently, Atkin et al. [8] had described that the direct correlation between TS expression and HIF-1α expression was recognized in primary rectal cancer, and the microenvironmental factor, such as acidosis or alternations in the availability of glucose and other enzymatic substrates, are more active in human cancers, thereby affecting the level of TS or HIF-1α expression. Thymidine phosphorylase is an enzyme involved in the activation and the metabolism of the

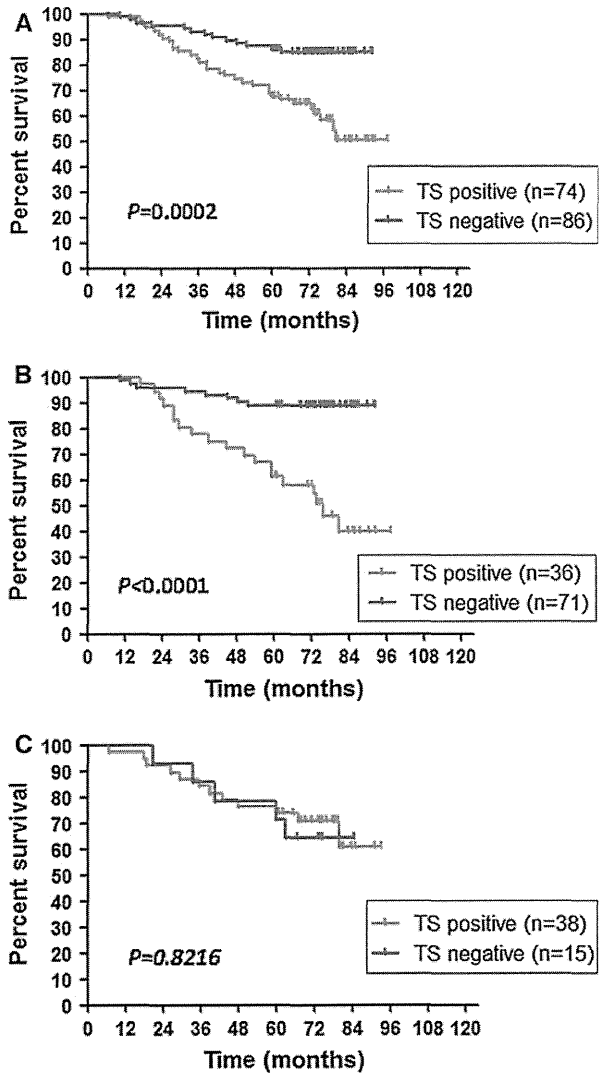

Fig. 2 Overall survival curve according to TS expression in all patients ($n = 160$) (**a**), patients with adenocarcinoma ($n = 107$) (**b**) and patients with squamous cell carcinoma ($n = 53$) (**c**)

fluoropyrimidines, including 5-FU, and previous report documented thymidine phosphorylase induction by hypoxia in a breast carcinoma cell line [16]. These reports including our study suggest that TS expression is related to hypoxia in cancer patients with a histological type of adenocarcinoma. Glut 1 is thought to be a possible intrinsic marker of hypoxia, and the expression of Glut 1 has been found to be regulated by hypoxia in HIF-1α [17, 18]. HIF-1α is considered to support tumor growth by the induction of angiogenesis via the expression of the VEGF and also by high and anaerobic metabolic mechanisms [19]. The overexpression of Glut1 has been shown to be closely related to 2-[^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) uptake in human cancer [11]. Previous studies suggest that hypoxic conditions correspond to a higher ^{18}F-FDG uptake [11]. In addition, there are also reports on the relationship between ^{18}F-FDG uptake and the expression of VEGF or MVD [11, 20]. These biomarkers including ^{18}F-FDG uptake have been documented to be closely associated with tumor aggressiveness and outcome in patients with NSCLC. In our study, we found that TS expression was a most powerful prognostic factor among these molecular markers in pulmonary AC patients. In non-AC patients, however, the direct relationship between TS expression and hypoxia was not observed, and TS expression was not related to the outcome after surgery. As TS expression in AC patients was closely correlated with the hypoxic and angiogenic markers (Glut1, HIF-1α and MVD) related to poor outcome, the expression of TS may play an important role for the prognosis and tumor aggressiveness of AC patients, but not non-AC (almost SQC).

It is noteworthy that adjuvant chemotherapy with UFT could improve survival in Japanese patients with completely resected NSCLC [21]. Adjuvant UFT was effective only in patients with early-stage adenocarcinoma, and no significant survival benefit was recognized in patients with SQC. Although these studies did not evaluate the TS expression in tumor specimens, TS inhibitor seems to be effective for the patients with pulmonary adenocarcinoma. Several studies have documented the clinical relevance of low TS expression as a predictor of the response to 5-FU-based chemotherapy and the long survival of the patients with advanced colorectal [22]. The results of these reports suggest that high expression of TS may be resistant to 5-FU-based chemotherapy in human neoplasms. However, it is unclear whether the expression level of TS is closely correlated with the chemoresistance to TS inhibitor regimens. On the other hand, several investigators have documented the relationship between TS expression and 5-FU-based chemotherapy in SQC [22]. In patients with oral SQC, the response to chemotherapy was higher in the group with low TS activity compared with high TS activity [23]. In contrast, high expression of TS in patients with esophageal SQC was associated with a higher response to chemotherapy than low expression of TS [24]. In our series, the patients with SQC had a high TS activity as compared with AC, but the TS expression seemed unlikely to be associated with tumor aggressiveness and metastasis. The prognostic significance of TS expression may be different between NSCLC patients with AC and SQC. Since a high TS expression in NSCLC patients with early-stage AC is associated with poor prognosis, it should be investigated whether the efficacy of UFT as adjuvant therapy is different according to a status of TS expression in patients with early-stage pulmonary AC.

S-1 (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan) is an oral anticancer agent comprised of tegafur (FT), 5-chloro-

Table 3 Univariate and multivariate analyses in the OS

Different variables	Univariate analysis		Multivariate analysis
	5-year survival rate (%)	*P* value	*P value*
Age			
≤65/> 65 years	81.7/74.2	0.1743	
Gender			
Male/female	72.2/85.6	**0.0484**	0.0723
Stage			
I + II/III	84.1/46.4	**<0.0001**	**<0.0001**
Histology			
AC/non-AC	80.3/71.7	0.1494	
TS			
Positive/negative	67.5/86.1	**0.0002**	0.1345
OPRT			
Positive/negative	71.6/84.7	0.1536	
DPD			
Positive/negative	71.9/91.3	**0.0022**	0.1121
Glut1			
Positive/negative	67.4/90.1	**0.0002**	0.1931
HIF-1α			
Positive/negative	72.9/92.1	**0.0105**	0.6478
VEGF			
Positive/negative	73.5/81.3	0.1712	
CD34			
Positive/negative	68.3/86.4	**0.0015**	0.7449
EGFR			
Positive/negative	69.7/87.3	**0.0010**	0.1820
p-Akt			
Positive/negative	74.0/80.7	0.3210	
p-mTOR			
Positive/negative	76.0/79.7	0.6820	
P53			
Positive/negative	73.9/80.5	0.1693	

TS thymidylate synthase, *OPRT* orotate phosphoribosyltransferase, *DPD* dihydropyrimidine dehydrogenase, *AC* adenocarcinoma, *Glut1* glucose transporter 1, *HIF-1α* hypoxia inducible factor-1α, *VEGF* vascular endothelial growth factor, *EGFR* epidermal growth factor receptor, *p-mTOR* phosph-mammalian target of rapamycin

Bold denotes statistically significance value

Table 4 Univariate and multivariate analyses in the PFS

Different variables	Univariate analysis		Multivariate analysis
	5-year survival rate (%)	*P* value	*P value*
Age			
≤ 65/> 65 years	64.8/65.2	0.9477	
Gender			
Male/female	59.8/73.0	0.1488	
Stage			
I + II/III	74.2/21,4	**<0.0001**	**<0.0001**
Histology			
AC/non-AC	65.4/64.2	0.7127	
TS			
Positive/negative	48.6/79.1	**<0.0001**	**0.0409**
OPRT			
Positive/negative	57.9/73.6	0.1409	
DPD			
Positive/negative	56.1/86.9	**0.0005**	0.1655
Glut1			
Positive/negative	51.7/81.7	**<0.0001**	0.1218
HIF-1α			
Positive/negative	57.4/89.5	**0.0002**	0.3529
VEGF			
Positive/negative	56.3/73.8	**0.0253**	0.2632
CD34			
Positive/negative	51.9/77.8	**0.0002**	0.7952
EGFR			
Positive/negative	55.1/77.5	**0.0088**	0.8640
p-Akt			
Positive/negative	57.1/72.3	**0.0341**	0.1022
p-mTOR			
Positive/negative	63.5/67.2	0.4137	
P53			
Positive/negative	57.5/71.3	0.0846	

TS thymidylate synthase, *OPRT* orotate phosphoribosyltransferase, *DPD* dihydropyrimidine dehydrogenase, *AC* adenocarcinoma, *Glut1* glucose transporter 1, *HIF-1α* hypoxia inducible factor-1α, *VEGF* vascular endothelial growth factor, *EGFR* epidermal growth factor receptor, *p-mTOR* phosph-mammalian target of rapamycin

Bold denotes statistically significance value

2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo), in a molar ratio of 1:0.4:1 [25]. S-1 is a potent inhibitor of DPD inhibitory fluoropyrimidine (DIF) and is effective against patients with lung, colon and gastric cancers [4, 26]. Recently, S-1 had been documented to be no difference in response and outcome between AC and SQC in advanced NSCLC [27]. However, UFT has been described to be effective in early-stage NSCLC, demonstrating a statistical significant difference between AC and SQC [21]. Our results indicated that TS and DPD expression were a significant prognostic factor in early-stage NSCLC but not in advanced stage NSCLC. The role of TS expression may be different according to not only histological type but also disease stage. Okabe et al. [9] described that inhibition of EGFR induced the down-regulation of TS expression in the experimental study. Our study indicated that TS expression was associated with EGFR/Akt/mTOR pathway in AC patients. The activation of this signaling pathway is related to tumor growth, and therefore, it may play a crucial role on the overexpression of TS in patients with AC. Recently, Fukui et al. [28]

reported the large-scale population study of TS, OPRT and DPD mRNA and protein expression in various cancers from a large number of subjects. High DPD expression was observed in most gastric, lung and pancreatic cancers, and 5-FU-resistant cancers exhibited high expression levels of DPD. Therefore, they described that the tumoral DPD level may be an important factor in predicting the effectiveness of 5-FU-based chemotherapy. The results of our study also demonstrated that a positive expression of DPD was closely related to poor outcome, especially in AC patients. Takeda et al. [29] had documented that a low expression level of TS or of DPD was associated with a better response and longer survival in advanced NSCLC patients treated with chemotherapeutic regimens including S-1, and TS or DPD expression was considered as the predictive biomarkers of S-1 treatment. Although their study included a small sample size ($n = 22$), 16 (73%) patients had AC, 1 patient SQC and five patients other histology. Their preliminary study suggests that TS expression seems to be a predictive marker for S-1 treatment in patients with AC. The expression of TS seems to be closely related to that of DPD in patients with AC. On the other hand, Sun et al. [30] described the clinical significance of TS expression in 193 patients with advanced non-squamous NSCLC treated by pemetrexed-based chemotherapy. Higher response rates for pemetrexed-based chemotherapy were associated with TS negativity (33.7% vs. 14.1, $P = 0.002$), and progression-free survival for pemetrexed-based chemotherapy was significantly longer in groups with TS negativity (4.1 vs. 2.0 months, $P = 0.001$). These reports suggest that low TS expression was associated with better clinical outcome in advanced pulmonary AC patients who were treated with S-1 or pemetrexed-based chemotherapy. Although the majority of our study was not treated by 5-FU or pemetrexed-based chemotherapy, our results indicated that TS protein expression could be a prognostic and predictive marker for predicting outcome after surgical treatment in patients with pulmonary AC. Nowadays, a low TS expression may be a better prognostic marker for predicting a favorable outcome after TS-targeting therapy in patients with advanced pulmonary AC. However, it remains unknown whether TS expression could be a clinical marker for predicting outcome after adjuvant therapy by 5-FU-based chemotherapy such as UFT in completely resected NSCLC patients with early-stage AC. Therefore, further prospective large-scale study is warranted to evaluate the prognostic significance between TS expression and 5-FU-based adjuvant chemotherapy after complete resection.

In conclusion, a positive TS expression was an independent prognostic factor to predict a poor outcome in completely resected NSCLC. When stratified according to histology, TS expression was closely associated with outcome, disease stage, tumor aggressiveness and hypoxia-related protein expression in patients with AC. In patients with early stage, TS and DPD expression seem to be related to a significant factor in predicting prognosis. Although TS expression was significantly higher in non-AC (almost SQC) than AC, the expression of TS in non-AC did not have an important role as the measurement of prognosis or tumor aggressiveness. In future, we will investigate the role of TS expression as a predictive and prognostic marker after 5-FU-based adjuvant chemotherapy in completely resected patients with early-stage pulmonary AC.

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