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Impact of thymidylate synthase protein expression on efficacy of chemotherapy in advanced lung cancer patients

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Abstract. Advanced lung cancer is primarily treated with platinum combination chemotherapy, however, its prognosis remains poor. The aim of this study was to examine the correlation between the expression of thymidylate synthase (TS) in cancer tissues and the efficacy of chemotherapy in patients with advanced lung cancer in order to clarify the role of TS in the overall response. In total, 120 patients diagnosed with lung cancer between June 2004 and December 2010 at Nihon University Hospital, Tokyo, Japan, were included in this study. Cancer tissue specimens were obtained from the included patients by surgery or bronchofiberscopy prior to treatment. The expression of TS protein was evaluated using specimens immunostained with anti-TS antibody and H-scoring. TS protein expression tended to be higher in smokers compared with non-smokers. Overall survival (OS) (median value) was significantly prolonged in the low TS expression group compared with the high TS expression group. More favorable therapeutic effects were observed in the high TS expression group compared with the low TS expression group, when carboplatin + paclitaxel combined chemotherapy (CbPac therapy) was used. When the therapeutic effects were compared between CbPac therapy and carboplatin + pemetrexed combined chemotherapy (CbPem therapy) in the high TS expression group, prolongation of OS (median value) was observed with CbPac therapy. The present study suggests that TS protein expression is a critical factor in determining the efficacy of CbPac therapy in lung cancer. CbPac therapy is more effective when TS protein is highly expressed in lung cancer tissue.

Introduction

Lung cancer has been the leading cause of mortality from cancer in Japan since 1998, with ~50,000 deaths annually. Anti-smoking measures as a primary prevention strategy, early diagnosis through health-check campaigns, as well as advances in surgical therapy, chemotherapy and radiation therapy have improved overall prognosis. However, the 5-year survival rate remains at only ~13%. Compared with other types of cancer, sufficient lung cancer therapeutic results have yet to be achieved. Lung cancer is frequently diagnosed in advanced stages, thus, the primary treatment mainly comprises platinum combination chemotherapy. Carboplatin + paclitaxel combination chemotherapy (CbPac therapy) is a typical regimen for non-small cell lung cancer (NSCLC), and is widely used in clinical practice.

The occurrence, progression and metastasis of cancer involve various gene and protein abnormalities. In lung cancer, mutations of the epidermal growth factor receptor (EGFR) and KRAS genes as well as abnormalities in protein expression have been previously reported (1-3). Recently, the correlation of these gene mutations and protein expression abnormalities with the therapeutic effects has been extensively studied, and EGFR gene mutation was identified as a predictive factor of the therapeutic effect of EGFR tyrosine kinase inhibitors (4,5).

Thymidylate synthase (TS) is a key enzyme in DNA synthesis and cell growth that has been suggested to be involved in malignancy (6-8). Moreover, it is a main target protein of antimetabolites, such as the anticancer agents pemetrexed (Pem) (9) and S-1 (10), demonstrating clinical efficacy in NSCLC. In colon (11), breast (12) and pancreatic cancer (13), an association between TS expression in cancer tissue, antitumor effects of chemotherapy and prognosis has been suggested. In lung cancer, a correlation between TS expression and prognosis has been suggested in early cancer (14). A limited number of studies have examined TS expression in advanced lung cancer, however, its impact on clinical effects remains to be determined. In particular, whether TS expression in cancer tissue is involved in the efficacy of CbPac therapy and prognosis remains to be elucidated. The aim of this study was to examine TS expression in cancer tissues obtained from patients with advanced lung cancer, and investigate the correlation between the expression rate and therapeutic effects and prognosis.

Patients and methods

Patients. In total, 120 patients diagnosed with lung cancer at Nihon University Hospital (Tokyo, Japan) between June 2004

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Table I. Patient characteristics and treatment methods.

Characteristics	Value, n (%)
Age (years)	
Mean (range)	65.7 (23-85)
Gender	
Male	78 (65.0)
Female	42 (35.0)
Histology	
Adenocarcinoma	81 (67.5)
Squamous cell carcinoma	17 (14.2)
Non-small cell carcinoma	12 (10.0)
Large cell carcinoma	7 (5.8)
Small cell carcinoma	3 (2.5)
EGFR mutation	
Positive	11 (9.2)
Negative	58 (48.3)
Unknown	51 (42.5)
ECOG performance status	
0	32 (26.7)
1	71 (59.2)
2	12 (10.0)
3,4	5 (4.2)
Stage of disease	
I, II	8 (6.7)
IIIA	12 (10.0)
IIIB	27 (22.5)
IV	73 (60.8)
Smoking status	
Former/current smoker	89 (74.2)
Non-smoker	29 (24.2)
Unknown	2 (1.7)
TS protein expession	
High	66 (55.0)
Low	54 (45.0)
Treatment	
Operation	7 (5.8)
Thoracic radiotherapy	2 (1.7)
Chemotherapy plus radiotherapy	23 (19.2)
Chemotherapy	
CbPac	50 (41.7)
Gemcitabine	2 (1.7)
Cisplatin + S-1	1 (0.8)
Docetaxel	1 (0.8)
Gefitinib	3 (2.5)
Carboplatin + Irinotecan	2 (1.7)
Carboplatin + gemcitabine	2 (1.7)
Carboplatin + PMT	14 (11.7)
Cisplatin + VNR	1 (0.8)
Irinotecan	1 (0.8)
PMT	1 (0.8)
Chemotherapy	
TS1	4 (3.3)
VNR	3 (2.5)
Others	3 (2.5)

Total no. of patients =120. EGFR, epidermal growth factor receptor; ECOG, Eastern Cooperative Oncology Group; TS, thymidylate synthase; CbPac, carboplatin + paclitaxel combined chemotherapy; PMT, Pemetrexed; VNR, vinorelbine.

and December 2010 were included in this study. Cancer tissue specimens were obtained from the included patients prior to treatment. The method of this study was approved by the ethics committee of Nihon University School of Medicine. Written informed consent was obtained from each subject. Cancer tissue specimens were collected by surgical procedure or bronchofiberscopic biopsy, then fixed in formalin and embedded in paraffin. Immunostaining was performed to examine the expression of TS protein. Patient background information is provided in Table I. The patients comprised 78 males and 42 females (mean age, 65.7 years). Additionally, there were 81 patients with adenocarcinoma, 17 with squamous cell carcinoma, 12 with non-small cell carcinoma, 7 with large cell carcinoma and 3 with small cell carcinoma. Eleven patients were positive for the EGFR gene mutation; performance status (PS) was 0-1 in 103 patients; the disease stage was IIIB or IV in 100 patients; and there were 29 non-smokers. The primary treatment for 85 patients (71%) was chemotherapy alone, and out of these 85 patients, 50 were administered CbPac combined chemotherapy (Table I).

TS immunostaining. Using the paraffin block of lung cancer tissue collected prior to treatment, the expression of TS protein was examined immunohistochemically. The paraffin block was cut into 10-μm sections and stained using immunostaining methods. The sections were then deparaffinized by being treated with 100% xylene three times for 2 min each, immersed in 99, 90 and 70% ethanol for 1 min each, washed with running water for 3 min and then reacted in 0.3% hydrogen peroxide-added methanol at room temperature for 15 min to inhibit endogenous peroxidase activity. After washing with water for 5 min, the sections were transferred to 0.01 M citrate buffer and treated in a microwave oven for 15 min to inactivate the antigen. After returning to room temperature, the sections were washed with 0.01 M phosphate-buffered saline (PBS), and allowed to stand in 2% bovine serum albumin (BSA)/PBS at room temperature for 15 min to inhibit non-specific reactions. The sections were then incubated with monoclonal mouse anti-TS antibody and diluted 100-fold with PBS as the primary antibody at 4˚C overnight. Subsequently, the sections were washed with PBS and incubated with peroxidase-labeled dextran 70-conjugated mouse immunoglobulin/goat anti-polyclonal antibody and peroxidase-labeled

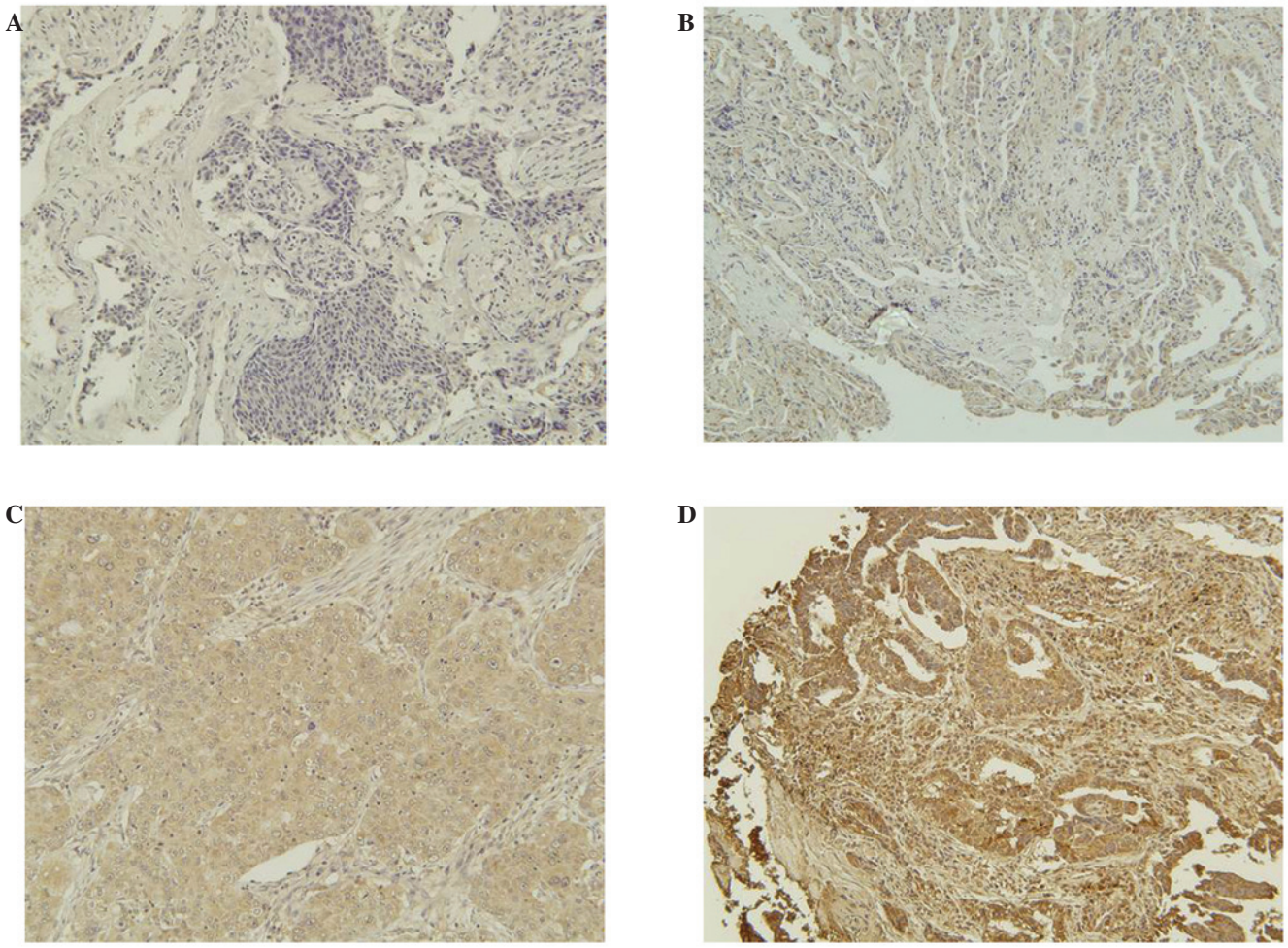

Figure 1. Immunohistochemical staining of human NSCLC tissue. Representative sections of carcinomas showing (A) negative staining, as well as (B) weak, (C) strong and (D) very strong expression of TS. Magnification, x200. NSCLC, non-small cell lung cancer.

dextran 500-conjugated anti-mouse immunoglobulin/goat anti-polyclonal antibody as the secondary antibodies at room temperature for 30 min.

After washing with PBS again, the sections were stained with 0.04% diaminobenzidine (DAB), nuclear stained with hematoxylin, washed with water, dehydrated with 70, 80, 90 and 99% ethanol, penetrated with 100% xylene, encapsulated and examined microscopically.

Evaluation of immunostaining. Expression of TS protein was evaluated using H-scoring. Staining intensity was evaluated as 0 (negative, Fig. 1A), 1 (weakly positive, Fig. 1B), 2 (moderately positive, Fig. 1C) or 3 (strongly positive, Fig. 1D), and multiplied by the proportion (%) of positive cells to calculate the H-score as previously described (15).

Statistical analysis. To examine the effect of patient background factors, the Mann-Whitney U test was used to compare TS protein expression in the two groups. To evaluate the correlation between TS protein expression and therapeutic effects, the latter were evaluated by investigating the response rate (RR), progression-free survival (PFS) and overall survival (OS). To compare RR between the two groups, the Chi-square test was performed, while the log-rank test was performed using the Kaplan-Meier method to compare PFS and OS between the two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TS protein expression and patient background factors. The mean H-score was 173.1±99.5 for adenocarcinoma, 200.0±71.5 for squamous cell carcinoma, 193.7±110.0 for large cell carcinoma, 188.7± ? for non-small cell carcinoma and 266.7±57.7 for small cell carcinoma patients. TS expression tended to be higher in small cell carcinoma compared with adenocarcinoma ($P=0.0974$), while no additional significant differences were observed among the other types of lung cancer tissue (Fig. 2A). Regarding the additional background factors, no significant differences in gender, presence/absence of EGFR gene mutation or PS were observed. However, the mean H-score was 150.7±87.2 for non-smokers and 192.3±97.6 for smokers, indicating a significantly higher expression of TS protein in smokers ($P=0.0429$) (Fig. 2B).

TS protein expression and patient survival. The median H-score in NSCLC patients was 200. Patients with a higher H-score were evaluated as the high TS protein expression

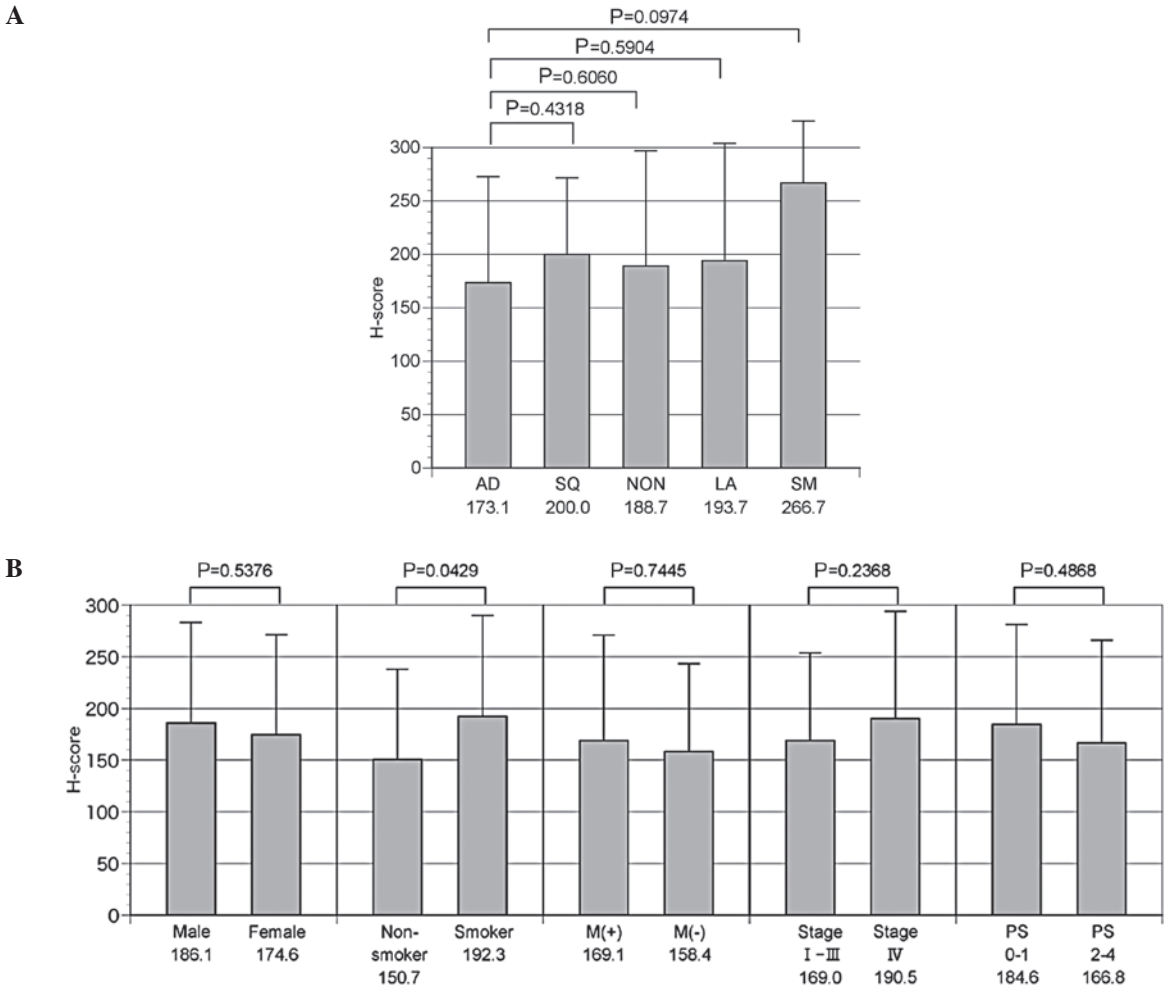

Figure 2. TS protein expression in lung cancer according to (A) tumor and (B) patient characteristics. AD, adenocarcinoma; SQ, squamous cell carcinoma; NON, non-small cell carcinoma; LA, large cell carcinoma; SM, small cell carcinoma; M, EGFR mutation; PS, performance status.

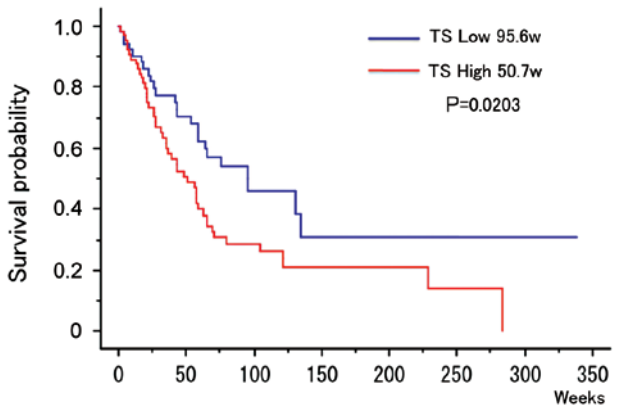

Figure 3. Thymidylate synthase (TS) protein expression of non-small cell lung cancer (NSCLC) according to overall survival.

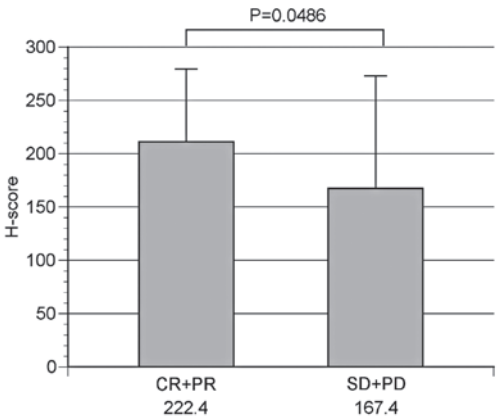

Figure 4. Response rate according to the expression level of TS in non-small cell lung cancer (NSCLC) in patients treated with CbPac. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

group (n=63, 53.8%) and patients with a lower H-score were evaluated as the low TS protein expression group (n=54, 46.1%). OS (median value) was 95.6 weeks in the low-expression and 50.7 weeks in the high-expression group, indicating a significant prolongation of survival in the low-expression group (P=0.0203) (Fig. 3).

TS protein expression and therapeutic effects. NSCLC was observed in 117 patients, 50 of whom were administered CbPac therapy as the primary treatment. The correlation between TS protein expression and the therapeutic effects of CbPac therapy was, therefore, examined in these 50 patients, 31 of whom (62.0%) comprised the high TS protein expression group and

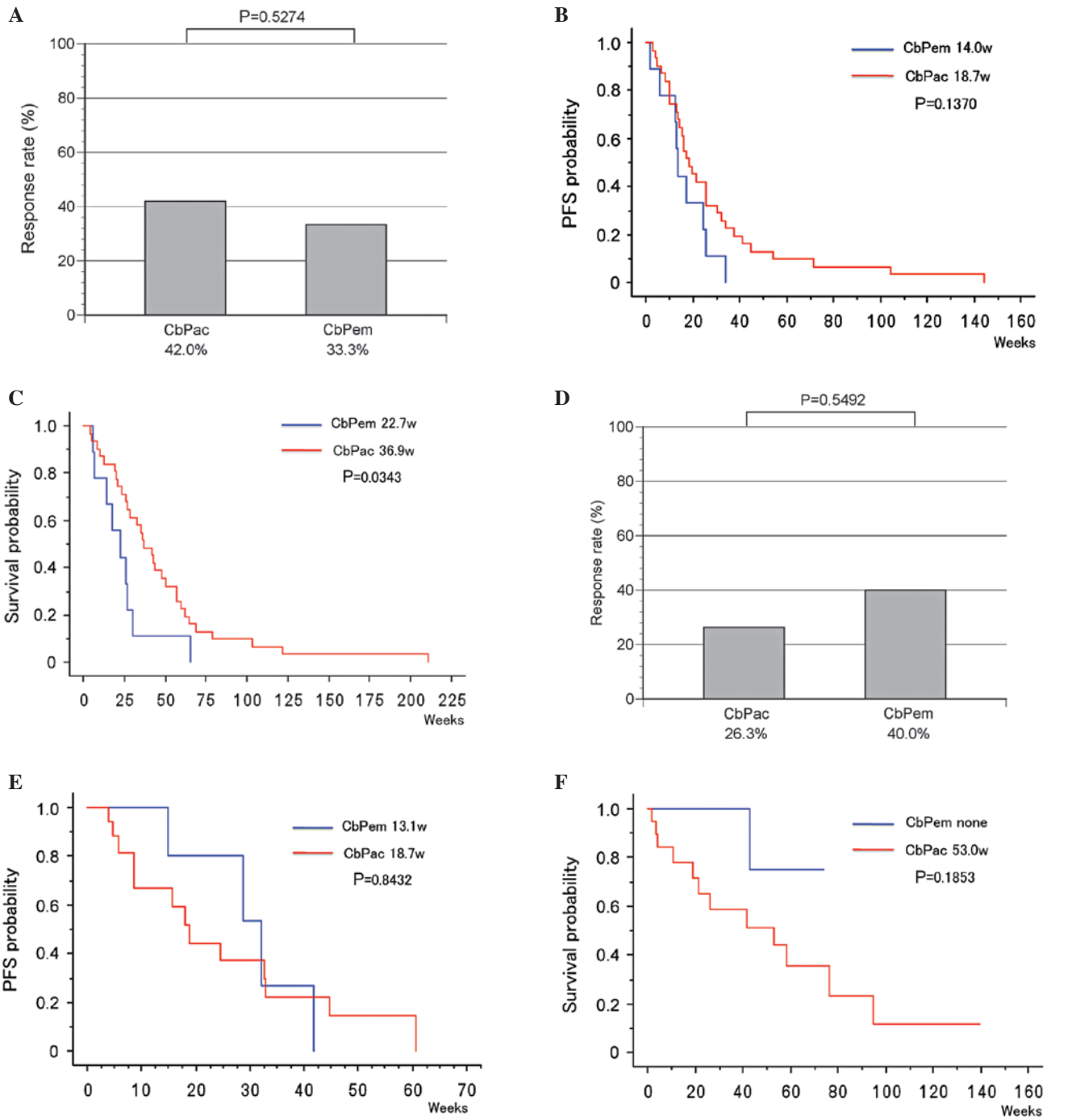

Figure 5. (A) Response rate, (B) progression-free survival and (C) overall survival in non-small cell lung cancer (NSCLC), indicating a high level of TS expression in patients treated with CbPac or CbPem. (D) Response rate, (E) progression-free survival (PFS) and (F) overall survival in NSCLC, indicating a low level of TS expression in patients treated with CbPac or CbPem. CbPac, carboplatin + paclitaxel combined chemotherapy; CbPem, carboplatin + pemetrexed combined chemotherapy.

19 (38.0%) the low TS protein expression group. Patients whose RR was complete response (CR) (n=0) or partial response (PR) (n=19) comprised the response group (n=19), while those whose RR was stable disease (SD) (n=20) or progressive disease (PD) (n=11) comprised the non-response group (n=31). The mean H-score was 222.4±68.1 in the response and 167.4±105.6 in the non-response groups, indicating a significantly higher TS expression in the response group (P=0.0486) (Fig. 4).

The therapeutic effects of CbPac (31 patients) and CbPem therapies (9 patients) as the primary treatment were compared in the high TS expression group of NSCLC patients. RR was 42.0% and 33.3% in the CbPac and CbPem therapy groups, respectively (P=0.5274, Fig. 5A). PFS (median value) was 18.7 and 14.0 weeks in the CbPac and CbPem therapy groups, respectively (P=0.1370, Fig. 5B). OS (median value) was 36.9 and 22.7 weeks in the CbPac and CbPem therapy groups, respectively, indicating a significant prolongation of OS in the CbPac therapy group (P=0.0343, Fig. 5C).

When CbPac (19 patients) and CbPem therapies (5 patients) were compared in the low TS expression group, RR was 26.3

and 40.0% in the CbPac and CbPem therapy groups, respectively (P=0.5492, Fig. 5D). PFS (median value) was 18.7 and 13.1 weeks in the CbPac and CbPem therapy groups, respectivley (P=0.8432, Fig. 5E). OS (median value) was 53.0 weeks in the CbPac therapy group, whereas the median survival period was not achieved in the CbPem group (P=0.1853, Fig. 5F).

Discussion

The standard treatment for advanced NSCLC is two-drug combination therapy containing a platinum agent, which has been shown to prolong the median survival time from 6 to 8 weeks and improve the 1-year survival rate from 15 to 25%, as previously demonstrated (16). One of the recommended drugs for combination with platinum is Pac, with CbPac therapy constituting one of the standard therapeutic methods for the treatment of lung cancer. Chemotherapy is employed in the treatment of advanced NSCLC. Thus, to increase its efficacy through the selection of the most suitable drugs, predictive factors are being investigated. One predictive factor of the efficacy of platinum agents is the excision repair cross-complementing 1 (ERCC1) gene, a DNA repair protein (17). Class III β-tubulin is another known prognostic factor of Pac (18). However, whether ERCC1 and β-tubulin are also predictive factors of the effects of CbPac therapy on advanced NSCLC remains to be determined.

TS metabolizes 5,10-methylenetetrahydrofolate (CH_2THF) as well as deoxyuridylate-5'-monophosphate (dUMP) by reductive methylation to deoxythymidine-5'-monophosphate (dTMP) to produce the thymine nucleotides required for DNA synthesis, and is involved in the biosynthesis of pyrimidine. 5-fluorodeoxyuridine monophosphate (FdUMP), a target enzyme of 5-fluorouracil (5-FU) and an active metabolite of 5-FU, binds to TS and activates folic acid to promote the formation of TS-FdUMP-CH_2FH_4 ternary complexes. When TS is completely inhibited, DNA synthesis in cells is also inhibited, resulting in antitumor effects. Accordingly, it has been reported that when the amount of TS protein in tumor cells is high, the antitumor effect of 5-FU is low, and when the amount is small, the sensitivity of 5-FU is high (19). In gastric (19), colon (11), breast (12) and pancreatic cancer (13), the correlation between TS expression and the therapeutic effects of 5-FU and its prognosis has been reported. Additionally, TS protein is positioned downstream of the cell growth signal and is involved in the proliferation of cancer cells. Therefore, the expression of TS protein in cancer cells may influence the effects of various anticancer drugs.

In the present study, we examined the correlation between the expression of TS protein, tissue type, patient background factors, prognosis and the therapeutic effects of CbPac therapy in lung cancer. No significant difference between tissue type and expression of TS protein was identified. However, the TS protein expression was higher in small and squamous cell carcinoma compared with adenocarcinoma, consistent with a previous study (20). Regarding the correlation between patient background factors and TS protein expression, there was no significant difference in gender, smoking status, EGFR gene mutation, clinical stage or PS, while TS protein expression was higher in smokers compared with non-smokers. Since smoking has been shown to induce mutations in genes such as p53 and KRAS (21-23), it is likely that this increase in TS protein expression was the result of a gene mutation induced by smoking. In addition, significant prolongation of OS was observed in the low compared with the high TS expression group, suggesting that TS protein expression may affect the prognosis of lung cancer. The rate of expression of TS protein in the primary lesion has been reported to correlate with malignancy (24), and is also thought to be involved in the biological malignancy of cancer.

The investigation of the correlation between the therapeutic effects of CbPac therapy and expression of TS protein demonstrated a higher TS in the response compared with the non-response group. This suggests that a higher TS expression is closely associated with higher efficacy of CbPac therapy. Moreover, when the effects of CbPac and CbPem therapies were compared in the high TS expression group, RR tended to be higher and PFS longer in the CbPac therapy group. OS was also significantly prolonged in the CbPac therapy group. These results suggest that Pac is more effective compared with Pem in the treatment of NSCLC with high TS expression, suggesting a higher efficacy of CbPac therapy in the high TS expression group, which is in contrast to the correlation between the amount of TS protein and the antitumor effect of 5-FU. As mentioned previously, 5-FU inhibits DNA synthesis to achieve antitumor effects. Similarly, Pem also inhibits DNA synthesis by inhibiting TS, a folate metabolic enzyme, to achieve antitumor effects. However, Pac stops cell division resulting in antitumor effects by inhibiting and stabilizing depolymerization of microtubules in the M phase of cell division. Since TS is a rate-limiting enzyme of DNA synthesis, a high TS expression is thought to indicate a high rate of cell division.

When CbPac and CbPem therapies were compared in the low TS expression group, RR was higher, while PFS and OS tended to be prolonged in the CbPem therapy group. In the low TS expression group, Pem was more effective compared with Pac. It has been reported (20) that Pem inhibits the growth of tumor cells and induces cell death mainly through the inhibition of TS, resulting in antitumor effects. Pem also shows high sensitivity in low TS-expressing cells. Therefore, Pem likely has an impact on NSCLC with low TS expression.

In the present study, expression of TS protein was examined using immunostaining methods. While quantification of mRNA can also be used to examine protein expression, immunostaining requires few specimens and is a simple procedure, constituting a convenient test method in clinical practice. Currently, the standard therapeutic method for primary treatment of NSCLC is CbPac + bevacizumab or cisplatin (Cis) + Pem. Therefore, prediction of the therapeutic effects of Pac and Pem is of high clinical relevance. Concerning the results of the present study, determination of TS protein expression using immunostaining methods is considered useful for the selection of Pac- or Pem-based platinum doublet during treatment. The findings also suggest that the selection of an effective primary treatment according to the rate of TS protein expression in lung cancer tissues may lead to improved prognosis, as observed in EGFR gene mutation analysis.

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