

Association between Expression of Thymidylate Synthase, Dihydrofolate Reductase, and Glycinamide Ribonucleotide Formyltransferase and Efficacy of Pemetrexed in Advanced Non-small Cell Lung Cancer

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Abstract. *Background:* Pemetrexed inhibits three key folate enzymes: thymidylate synthetase (TYMS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). The relationship between the clinical efficacy of pemetrexed and the expression of folate enzymes in lung cancer cells is unknown. The purpose of this study was to determine whether TYMS, DHFR, and GARFT expression affect the therapeutic efficacy of pemetrexed. *Patients and Methods:* Participants (n=50) were patients with advanced non-small cell lung cancer (NSCLC) treated with pemetrexed. Samples were obtained by tumor biopsy before treatment. We isolated cancer cells from formalin-fixed paraffin-embedded tissues using laser microdissection, and mRNA levels were analyzed using real-time reverse transcription polymerase chain reaction. Protein expression was evaluated using immunohistochemistry. We assessed the association between TYMS, DHFR, and GARFT expression and the therapeutic efficacy of pemetrexed. *Results:* The median age was 66.8 years. Compared to healthy tissues, the relative TYMS mRNA expression ranged from 0.001 to 41.613 (mean 4.638 ± 1.357), and was significantly lower in responders compared to non-responders (1.671 ± 0.844 versus 5.978 ± 1.895 , $p=0.0142$). Progression-free survival was prolonged in patients with lower TYMS mRNA expression compared to those with higher TYMS mRNA expression, but

the difference was not statistically significant (18.0 versus 13.3 weeks, $p=0.3001$). DHFR and GARFT mRNA expression did not correlate with the efficacy of pemetrexed. *Conclusion:* We specifically analyzed TYMS, DHFR, and GARFT mRNA expression levels in lung cancer cells from biopsy specimens using laser microdissection. TYMS mRNA expression affected the therapeutic efficacy of pemetrexed and could therefore constitute a useful predictive biomarker for NSCLC patients receiving pemetrexed.

Pemetrexed is an effective drug for the treatment of non-small cell lung cancer (NSCLC) and malignant mesothelioma. In a phase III study of treated patients with NSCLC, pemetrexed had a similar efficacy and safety profile as monotherapy, compared with docetaxel (1). In another phase III study of chemotherapy-naïve patients with NSCLC, cisplatin plus pemetrexed had non-inferior efficacy and better tolerability than cisplatin plus gemcitabine (2). Pemetrexed is widely used and is one of the standard therapeutic agents in the therapy of advanced NSCLC (3).

Pemetrexed, a multitargeted antifolate drug, inhibits three key folate-dependent enzymes: thymidylate synthase (TYMS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). TYMS is an important enzyme in DNA synthesis and repair; it converts deoxyuridine monophosphate to deoxythymidine monophosphate, and has a role in purine and pyrimidine synthesis (4). Pemetrexed potently inhibits TYMS among the three enzymes (5-8). It has been reported that overexpression of TYMS correlates with reduced sensitivity to pemetrexed *in vitro* (9).

In two large phase III NSCLC studies, pemetrexed was shown to be more effective in patients with adenocarcinoma than in those with squamous cell carcinoma (1, 2). Thus the efficacy of pemetrexed is dependent on histology (10). Ceppi *et al.* reported that TYMS mRNA and protein expression in

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surgical samples was significantly lower in adenocarcinoma compared with squamous cell carcinoma (11). This observation suggests that the efficacy of pemetrexed is related to TYMS expression. However, the relationship between the expression levels of these folate-dependent enzymes in lung cancer cells and the clinical efficacy of pemetrexed is controversial (12-16). In particular, the gene expression of folate enzymes in micro-biopsy samples from patients with advanced NSCLC is unknown. The purpose of the present retrospective study was to determine whether the expression of TYMS, DHFR, and GARFT affects the therapeutic efficacy of pemetrexed in patients with advanced NSCLC.

Patients and Methods

Patients. Fifty patients with advanced NSCLC who were treated at the Nihon University, School of Medicine (Tokyo, Japan) from July 2009 to November 2010, were examined. All patients received pemetrexed-based chemotherapy. Written informed consent was obtained from all participants.

Samples and microdissection. Samples were obtained by transbronchial or percutaneous tumor biopsy before treatment. Serial sections (thickness of 10 µm) were prepared from formalin-fixed paraffin-embedded (FFPE) microsamples and stained with toluidine blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Lung cancer cells were identified under a microscope and were isolated by a pathologist using laser microdissection.

RNA extraction and combinatorial DNA synthesis. Samples were heated at 55°C for 24 h with 5 µl Proteinase K (Takara Bio Inc., Shiga, Japan), then 20 µl of 2 M sodium acetate, followed by 220 µl phenol/chloroform/isoamyl alcohol (250:50:1 dilution) were added to the sample suspension. The suspension was then centrifuged at 10,000 ×g for 15 min. The supernatant was added to 2 µl glycogen and 200 µl isopropanol and stored at -80°C for 30 min. After centrifugation at 10,000 ×g for 30 min at 4°C, the supernatant was carefully removed. The pellet was resuspended in 5 µl RNase-free water. Combinatorial DNA was synthesized using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany).

Real-time reverse transcription-polymerase chain reaction. TYMS, DHFR, and GARFT cDNA sequences were amplified by quantitative polymerase chain reaction (PCR) using a fluorescence-based real-time detection method in duplicate, as previously described (11). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. The PCR mixture consisted of 1,200 nM of each primer, 200 nM probe, 200 nM each of dATP, dCTP, dGTP, and dTTP, 3.5 mM MgCl₂, and 1× Taqman Universal PCR Master mix in a final volume of 20 µl (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences were as follows: TYMS: forward: 5'-GAATCACATCGAGCCACTGAAA-3', reverse: 5'-TTCGAAGAATCCTGAGCTTTGG-3'; DHFR: forward: 5'-TAAACTGCATCGTCGCTGTGT-3', reverse: 5'-GGGCAGGTCCCCGTCT-3'; GARFT: forward: 5'-GACAGTACTCGGGAACCAAATAGC-3', reverse: 5'-ACTGCGGCTTTGTTGAGAT-3'; GAPDH: forward: 5'-GTCGGAGTCAACGGATTGG-3', reverse: 5'-GGCAACAATA TCCACTTTACCAGAG-3'. PCR conditions were 50°C for 2 min,

95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. The mRNA level in the samples for each gene was defined as the relative value compared with that of normal lung tissue.

TYMS protein expression analysis. A paraffin block of lung cancer tissue was cut into sections (10 µm in thickness) and immunostained to determine the protein expression. Sections were de-paraffinized by treatment with 100% xylene three times for 2 min, immersing in 99%, 90%, and 70% ethanol for 1 min, washing with running water for 3 min, before reacting with 0.3% hydrogen peroxide in 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 microwaved for 15 min to inactivate the antigen. After returning to room temperature, the sections were washed with 0.01 M phosphate-buffered solution (PBS), allowed to stand in PBS, containing 2% bovine serum albumin at room temperature for 15 min, to inhibit nonspecific reactions, and finally reacted with a monoclonal antibody against TYMS (Invitrogen, Camarillo, CA, USA) diluted 100-fold with PBS at 4°C overnight. Subsequently, the sections were washed with PBS and reacted with peroxidase-labeled dextran 70-conjugated mouse immunoglobulin/goat anti-polyclonal antibody and peroxidase-labeled dextran 500-conjugated anti-mouse immunoglobulin/goat polyclonal antibody (Dako North America Inc., CA, USA), as secondary antibodies at room temperature for 30 min. After washing with PBS again, the sections were colored with 0.04% diaminobenzidine, nuclear stained with hematoxylin, washed with water, dehydrated with 70%, 80%, 90%, 99%, and 99% ethanol, penetrated with 100% xylene, encapsulated, and microscopically examined. Expression of TYMS protein was evaluated using the immunohistochemistry (IHC) score, as described previously (11). The immune reaction was scored as low expression or high expression in a semiquantitative manner. The value of IHC score was defined as staining intensity (0, negative; 1, weakly-positive; 2, moderately-positive; 3, strongly-positive) multiplied by the proportion (%) of positive cells.

Statistical analysis. The Mann-Whitney U-test was used to determine the correlation between TYMS, DHFR, and GARFT mRNA levels and the response to pemetrexed, and also to test for the correlation between TYMS mRNA and protein expression, and between TYMS mRNA levels and patient/disease characteristics (sex, histology, stage). The Kaplan-Meier method was used to estimate the median values for progression-free survival (PFS) and overall survival (OS). The log-rank test was used to test for significant differences between the low- and high-mRNA expression groups for TYMS, DHFR, and GARFT. The level of significance was set at *p*<0.05. Analysis was performed using SPSS 11.0.1J (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics. Patients' characteristics are shown in Table I. The median age was 66.8 years, and there were 29 male and 21 female patients. Histologically, most of the patients had a diagnosis of adenocarcinoma. Only five patients had epidermal growth factor receptor (EGFR) mutation-positive tumors. Seventeen patients had not previously received chemotherapy; in the other 33 patients,

Table I. *Patients' characteristics.*

Characteristic	n=50
Age (years)	66.8 (40-85)
Gender (m/f)	29/21
Histology	
Adenocarcinoma	38
Squamous cell carcinoma	3
Non-small cell carcinoma	7
Large cell carcinoma	2
EGFR mutation-positive	5
Stage IIIA/IIIB/IV	3/9/38
Prior regimens	
0	17
1	12
2	8
3 or more	13
Response rate	30.0%
Progression-free survival	16.7 weeks

prior regimens were mostly taxanes and chemotherapy combined with radiotherapy. The overall response rate was 30% for pemetrexed. The median PFS was 16.7 weeks for pemetrexed.

TYMS mRNA expression affected response to pemetrexed. We examined the influence of *TYMS* mRNA expression on the therapeutic efficacy of pemetrexed. Relative *TYMS* mRNA levels ranged from 0.001 to 41.613 (mean 4.638 ± 1.357). Firstly, we analyzed the correlation between pemetrexed response and *TYMS* mRNA expression. Patients were assigned to two groups based on pemetrexed response: response group, patients with complete or partial response, and the non-response group, patients with stable or progressive disease. The mean *TYMS* mRNA levels were 1.671 ± 0.844 and 5.978 ± 1.895 in the response and non-response groups, respectively. *TYMS* mRNA expression was significantly lower in the response group compared with the non-response group ($p=0.0142$, Figure 1A). *DHFR* and *GARFT* mRNA expression levels were not correlated with response rate (Figure 1B and C).

Association of TYMS, DHFR, and GARFT with PFS and OS, following treatment with pemetrexed. We analyzed the association of PFS and OS following pemetrexed treatment with the mRNA expression of *TYMS*, *DHFR*, and *GARFT*. Patients were grouped according to their mRNA expression, using the mRNA level in normal lung tissue as a cut-off value. PFS was prolonged in patients with lower *TYMS* mRNA expression compared with those with higher *TYMS* mRNA expression, however, this difference was not statistically significant (18.0 versus 13.3 weeks, $p=0.3001$) (Figure 2). No correlation was observed between *DHFR* and

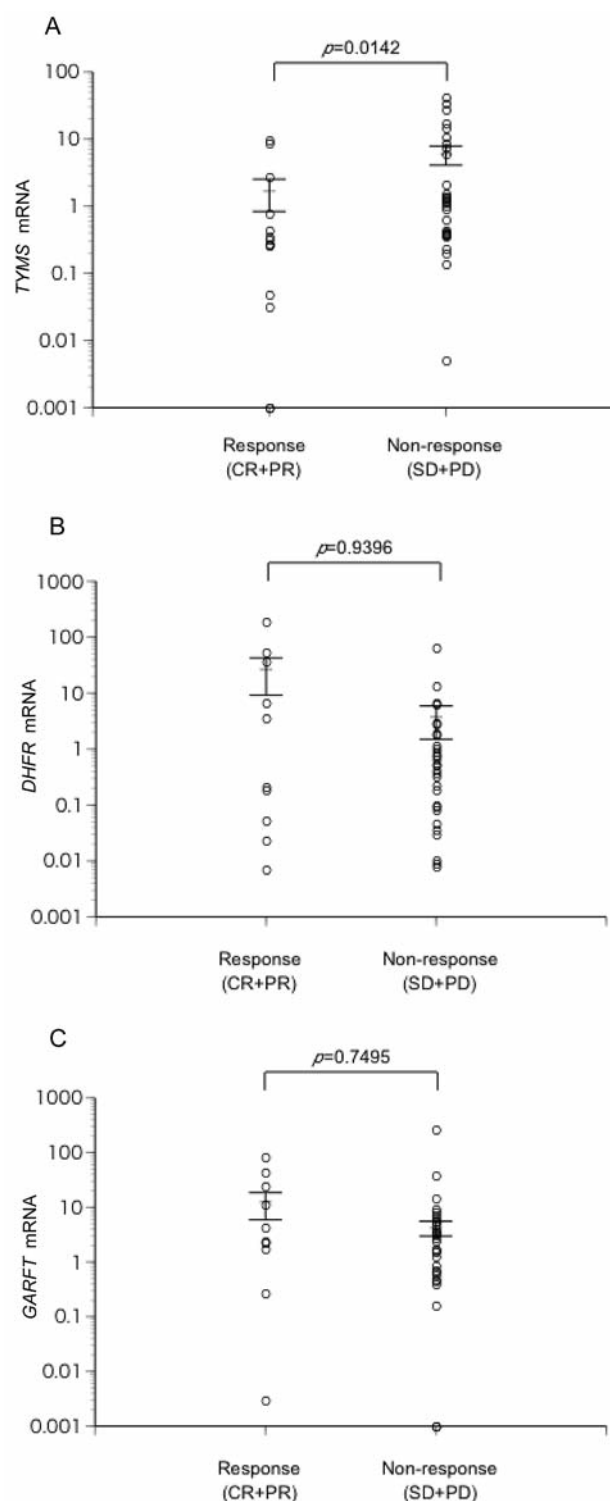


Figure 1. Association between mRNA levels of thymidylate synthetase (*TYMS*) (A), dihydrofolate reductase (*DHFR*) (B), and glycylamide ribonucleotide formyltransferase (*GARFT*) (C) in lung cancer cells and response to pemetrexed. Central horizontal lines represent median values, with the upper and lower bars representing the 95% confidence interval. CR: Complete remission; PR: partial remission; SD: stable disease; PD: progressive disease.

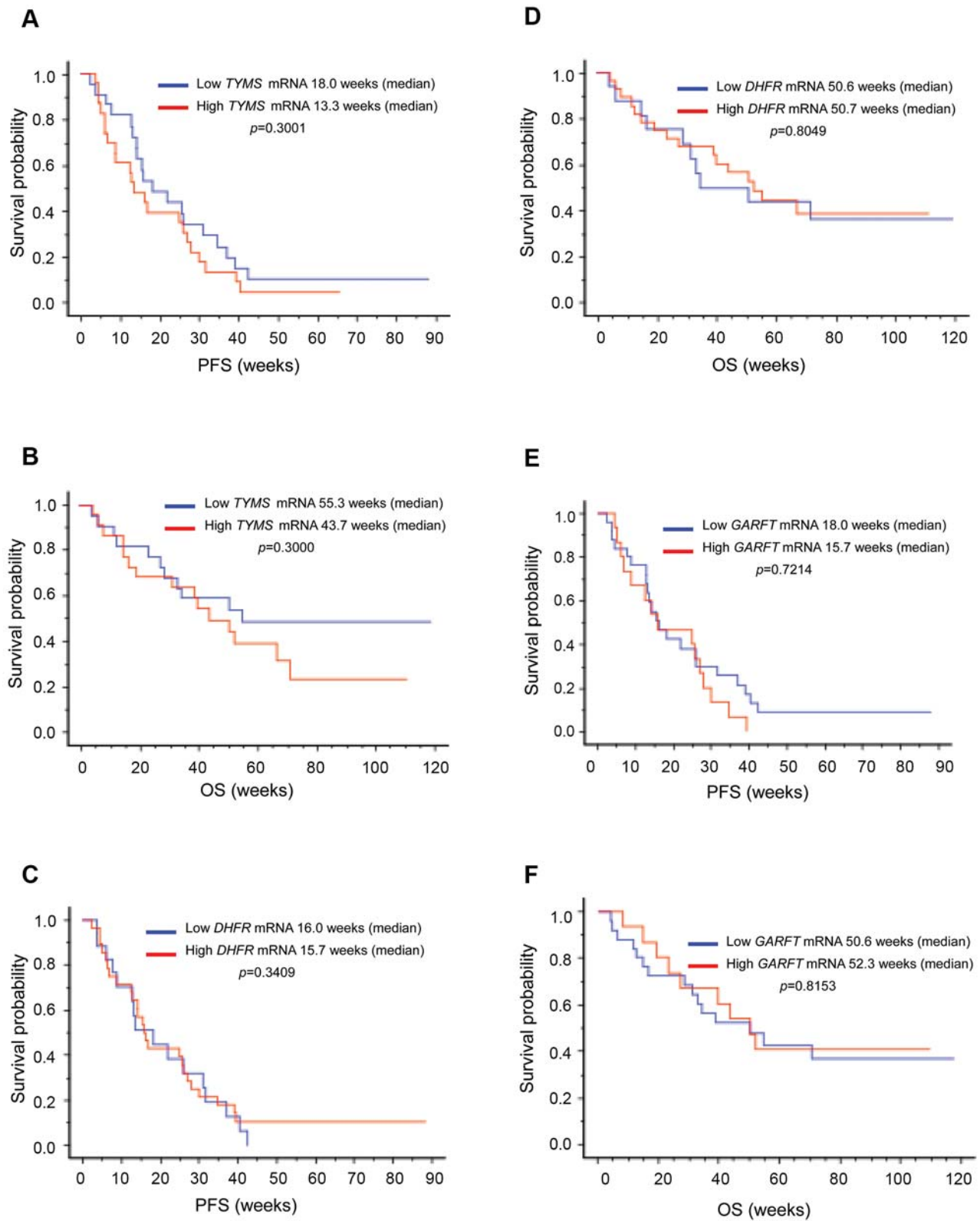


Figure 2. Kaplan–Meier progression-free survival (PFS) and overall survival (OS) curves for mRNA expression of thymidylate synthetase (*TYMS*) (A, B), dihydrofolate reductase (*DHFR*) (C, D), and glycylamide ribonucleotide formyltransferase (*GARFT*) (E, F) in lung cancer cells.

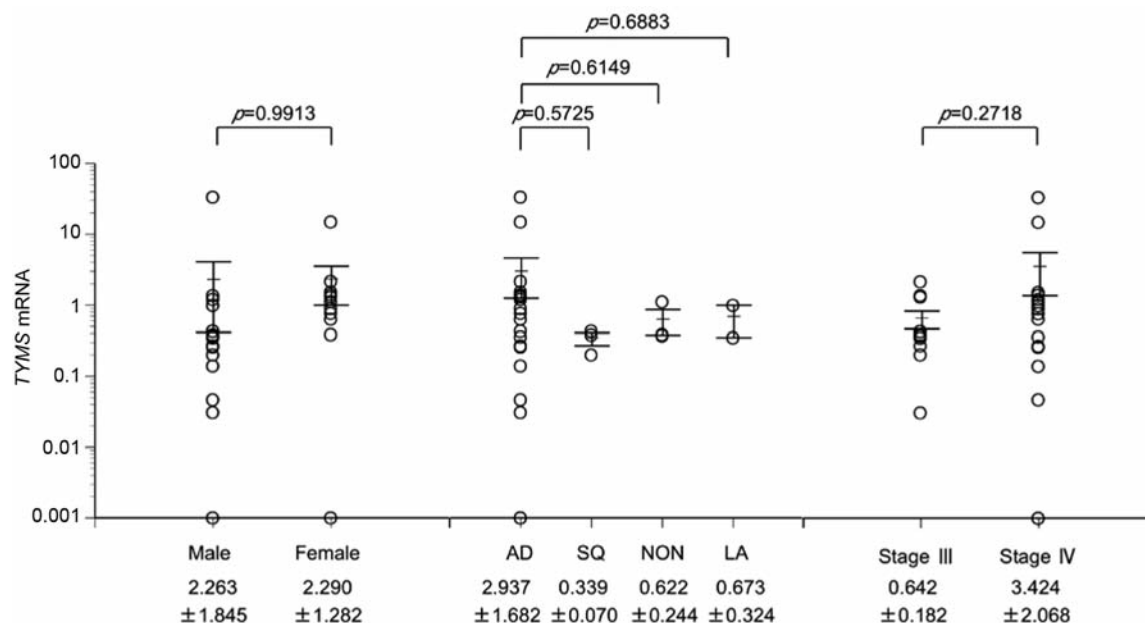


Figure 3. Thymidylate synthetase (TYMS) mRNA expression according to patients' characteristics. Central horizontal lines represent median values, with the upper and lower bars representing the 95% confidence interval. AD: Adenocarcinoma; SQ: squamous cell carcinoma; NON: non-small cell carcinoma; LA: large cell carcinoma.

GARFT mRNA expression levels and PFS, nor between mRNA expression levels of all three enzymes and OS.

TYMS, DHFR, and GARFT mRNA expression according to patients' characteristics. We examined the TYMS mRNA expression according to patient and disease characteristics (Figure 3). TYMS mRNA levels did not correlate significantly with sex, histology, or clinical stage. Furthermore, DHFR and GARFT expression levels did not correlate with these characteristics (data not shown). Three patients in this study had squamous cell carcinoma, and TYMS expression in these patients did not exceed the one of patients with adenocarcinoma. All patients with squamous cell carcinoma achieved stable disease, and PFS was not inferior for patients with squamous cell carcinoma compared with those with adenocarcinoma.

Association between TYMS protein expression and efficacy of pemetrexed. We analyzed the relationship between TYMS protein expression and its mRNA expression and found a significant correlation ($p=0.0299$) (Figure 4). Next, we examined the influence of TYMS protein expression on the therapeutic efficacy of pemetrexed. The response rate in the low-TYMS protein expression group was higher than that in the high-expression group, however, this difference was not statistically significant (37.5% versus 23.1%, $p=0.4543$).

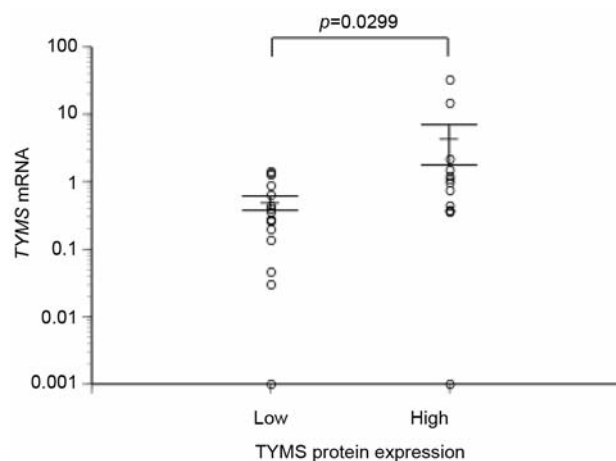
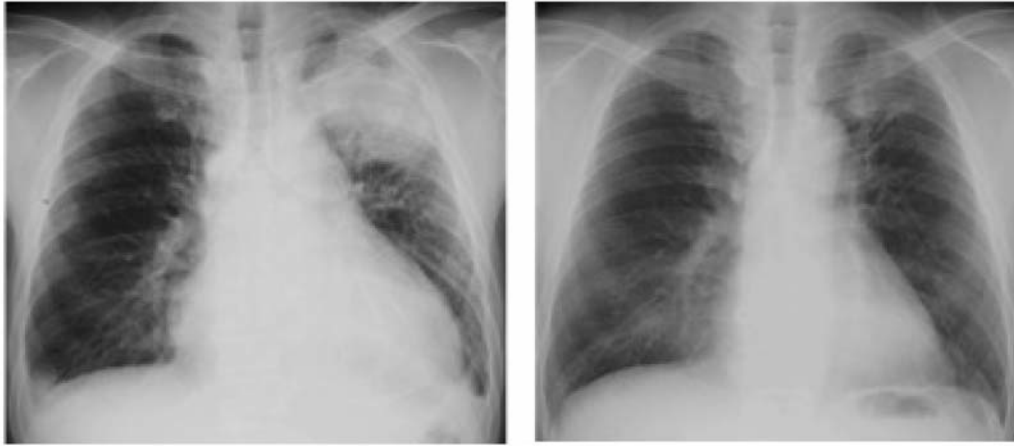


Figure 4. Correlation between thymidylate synthetase (TYMS) protein and mRNA expression. Central horizontal lines represent median values, with the upper and lower bars representing the 95% confidence interval.

Cases. Two representative cases are shown in Figure 5. The first case shows a 67-year-old man who had been diagnosed with EGFR mutation-negative adenocarcinoma of the lung. TYMS protein expression was low, and the TYMS mRNA level was also very low, at 0.004. Chest X-ray on admission showed a tumor shadow in the left upper lobe. Carboplatin and pemetrexed were given as first-line chemotherapy, and proved very effective in reducing tumor size. A partial

Case 1



Case 2

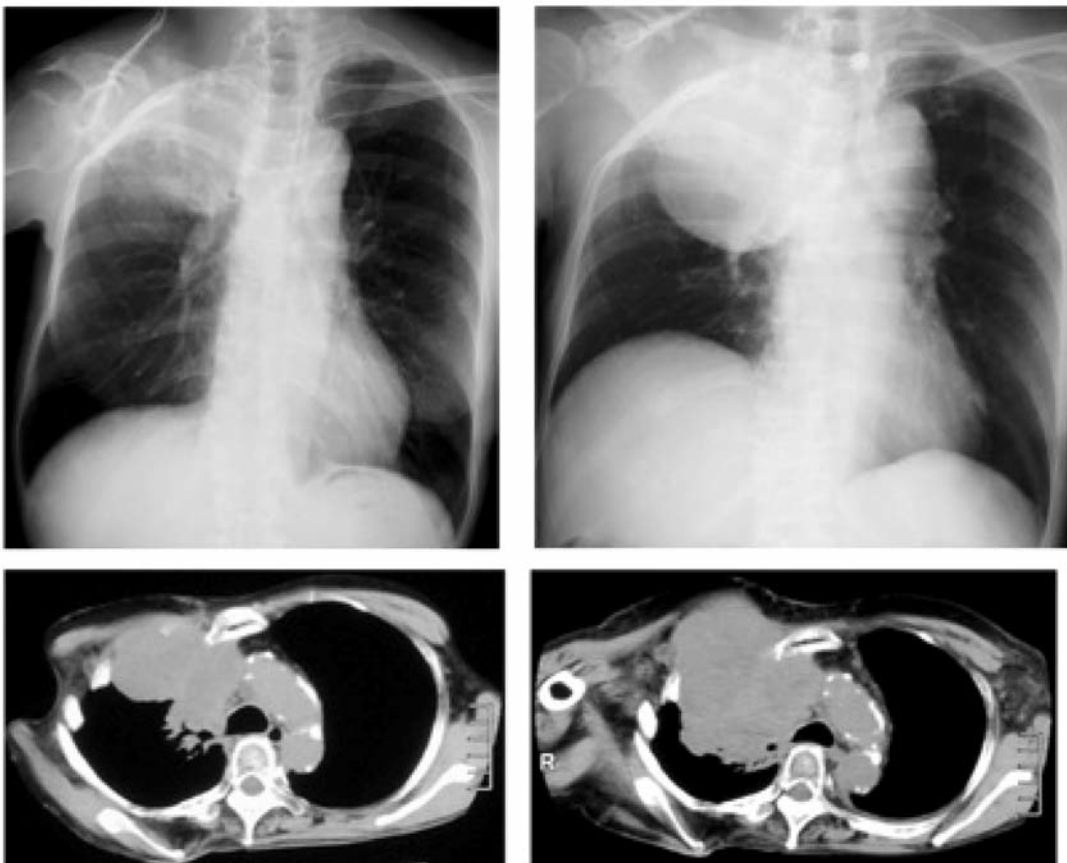


Figure 5. Case 1. A 67-year-old man, with, epidermal growth factor receptor (EGFR) mutation-negative adenocarcinoma. The expression of thymidylate synthetase (TYMS) mRNA was low (0.004). The left panel shows the chest X-ray on admission. The chest X-ray in the right panel shows the reduction in tumor size (partial response) after administration of pemetrexed. Case 2. A 50-year-old woman with EGFR mutation-negative adenocarcinoma. The expression of thymidylate synthetase (TYMS) mRNA expression was high (2.161). Left panel shows the chest X-ray and the computed-tomographic (CT) scan on admission. Chest X-ray and CT scan in the right panel show an increase in tumor size (progressive disease) after administration of pemetrexed.

response was achieved after four courses of therapy. The second case was a 50-year-old woman who had been diagnosed with *EGFR* mutation-negative adenocarcinoma of the lung. TYMS protein expression was high and the *TYMS* mRNA level was also high, at 2.161. Chest X-ray and computed-tomographic (CT) scan on admission showed a tumor shadow in the right upper lobe. The patient received carboplatin and pemetrexed as first-line chemotherapy, which were not effective in increasing tumor size. These cases indicate that TYMS expression influences the efficacy of pemetrexed in lung cancer patients.

Discussion

Pemetrexed targets folate-dependent enzymes to inhibit cancer cell proliferation. Overexpression of TYMS leads to reduced sensitivity to pemetrexed (17-19). Elevated activity of the target enzymes is one proposed mechanism of drug resistance. We believe that the activity of these folate enzymes is likely to be involved in the mechanism of resistance to pemetrexed, hence it is important to examine mRNA expression of these enzymes in patients with NSCLC, treated with pemetrexed. We specifically analyzed *TYMS*, *DHFR*, and *GARFT* mRNA expressions in lung cancer cells from FFPE specimens. Most samples were obtained by transbronchial tumor biopsy, and were consequently very small. Cancer cells are generally mixed with stromal cells and must be, therefore, isolated from samples in order to measure tumor-specific mRNA expression. It is easy to select the tumor area from surgically resected samples, but this is difficult in biopsy samples. We used laser microdissection to isolate lung cancer cells from microsamples, and we believe that this technique is essential for analyzing tumor-specific mRNA expression in microsamples.

IHC can also be used to analyze expression of these enzymes in cancer tissue and to detect protein by using specific antigens. In our study, TYMS protein expression correlated with *TYMS* mRNA expression. *TYMS* mRNA expression significantly correlated with response rate, but TYMS protein expression did not. The main reason for the discrepancy in these results is likely to be that IHC methods are inferior to quantitative mRNA measurements. Most cancer cells express TYMS because its protein is an E2F1-regulated enzyme that is essential for DNA synthesis and repair (20). We believe that quantitative examination is more suitable for analysis of TYMS compared with qualitative examination. With regard to measuring the expression of TYMS, analysis of mRNA is more useful than IHC.

We found that the level of *TYMS* mRNA expression was significantly correlated with response to pemetrexed treatment. PFS in patients with low-*TYMS* mRNA expression was superior to the one of patients with high-*TYMS* mRNA

expression. Accordingly, *TYMS* mRNA levels in lung cancer cells are strongly predictive of clinical outcome in patients receiving pemetrexed. It was similarly reported that TYMS predicts outcome in patients with malignant mesothelioma (21). It has also been demonstrated that TYMS expression is higher and that pemetrexed treatment is less effective in squamous cell carcinoma than in adenocarcinoma (22). However, patients with squamous cell carcinoma in the present study had low-*TYMS* mRNA expression and good outcome. We think it is possible that pemetrexed is effective in patients with squamous cell carcinoma with low-TYMS expression. It appears that *TYMS* mRNA expression is more useful than histology as a predictive marker of response to pemetrexed. *DHFR* and *GARFT* are secondary targets of pemetrexed (23, 24) and did not affect the efficacy of pemetrexed in our study. It appears that *DHFR* and *GARFT* expression cannot predict outcome in lung cancer patients receiving pemetrexed. Most patients received further chemotherapy after pemetrexed treatment. We suggest that post-pemetrexed treatment affects OS. Results revealed that neither *TYMS*, *DHFR*, nor *GARFT* affected OS. In addition, no significant association was observed between *TYMS* mRNA expression and sex, histology, or clinical stage. These results suggest that TYMS expression is not influenced by patient/disease characteristics.

We demonstrated that *TYMS* mRNA expression in micro biopsy samples affects the efficacy of pemetrexed in patients with advanced lung cancer. Most patients with lung cancer have advanced-stage disease at the time of diagnosis. Therefore, analysis of lung cancer tissue commonly utilizes biopsy samples. As it is difficult to obtain large samples, it is important that microsamples are analyzed using laser microdissection.

In conclusion, our findings indicate that *TYMS* mRNA expression could be a useful predictive biomarker of therapeutic efficacy for patients with NSCLC receiving pemetrexed.

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