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Molecular Factors Associated with Pemetrexed Sensitivity According to Histological Type in Non-small Cell Lung Cancer

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Abstract. *Background: This study was designed to investigate potential molecules that predict chemosensitivity to pemetrexed (Alimta®) in surgically resected non-small cell lung cancer (NSCLC). Materials and Methods: Chemosensitivity to ALM and other drugs was assessed by succinate dehydrogenase inhibition (SDI) test in 69 NSCLC samples (55 adenocarcinomas, and 14 squamous cell carcinomas). The mRNA expression levels of Alimta®-target enzymes [thymidylate synthase (TYMS); dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltransferase (GARFT)], Alimta®-metabolizing enzymes [γ-glutamyl hydrase (GGH) and folylpolyglutamate synthase] and an Alimta® transporter [reduce folate carrier (RFC)] were measured and examined for potential correlations to chemosensitivity. Results: The squamous cell carcinoma samples showed higher TYMS expression and lower RFC expression than did the adenocarcinoma samples. In the adenocarcinoma sample analyses, GGH expression was inversely correlated to sensitivity. Conclusion: The histology-dependent differences in chemosensitivity to Alimta® may be attributed to the histology-dependent differences in TYMS and RFC expression. In adenocarcinomas, GGH potentially represents a marker for chemosensitivity to Alimta®.*

Lung cancer is currently the leading cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases (1). However, chemotherapy for advanced NSCLC remains a challenge since the efficacy of cytotoxic agents for NSCLC is limited; the median survival time for patients with stage IIIB-IV disease who receive chemotherapy is currently only 10 months to 1 year (2).

Pemetrexed (Alimta®) is a multi-targeted, folate anti-metabolite chemotherapy drug with effects demonstrated in NSCLC and other cancer types (3-6). A large phase III study demonstrated that Alimta® in combination with cisplatin provided similar efficacy and improved tolerability relative to treatment with gemcitabine in combination with cisplatin for patients with NSCLC (2). Recently, sub-analyses of three randomized phase III trials revealed that patients with non-squamous cell carcinoma (non-SCC) treated with Alimta® had better survival rates than those given alternative treatments (2, 7-9). Therefore, it is prudent to consider Alimta®-based treatment for patients with advanced non-SCC NSCLC. However, the mechanisms behind histologically dependent sensitivity to Alimta® are not fully understood. Some studies have attempted to identify molecular biomarkers that predict the efficacy and toxicity of Alimta®, irrespective of histological cell type (10-11). However, no additional predictive markers have been identified in the clinical setting to our knowledge.

Alimta® is considered to exert its antitumor effect by inhibiting several enzymes that participate in the thymidine and purine biosynthetic pathways, including thymidylate synthase (TYMS), dihydrofolate reductase (DHFR), glycinamide ribonucleotide formyl transferase (GARFT), and aminoimidazole carboxamide ribonucleotide-formyltransferase (12). Some studies have shown in various tumor cell lines, such as colon, breast and lung cancer, that higher gene or protein levels of TYMS are associated with resistance to Alimta® treatment (13, 14). In regard to lung cancer cell lines, TYMS expression was found to be lower in adenocarcinoma (AD) than in SCC (15). In addition, low TYMS levels were associated with better outcomes in patients with non-SCC treated with Alimta® than in patients with SCC (16).

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As a folic acid analog, Alimta® is taken up into the cell by transporters, such as the reduced folate carrier (RFC). Upon entry into the cell, the activity of Alimta® is dependent on its conversion into active polyglutamate derivatives, which is catalyzed by folylpolyglutamate synthase (FPGS). This step increases the intracellular retention of Alimta®, and thereby, the affinity of Alimta® for its intracellular targets by 50- to 100-fold. This polyglutamation step competes with the hydrolysis of the accumulated pentaglutamate tails catalyzed by γ-glutamyl hydrolase (GGH), which allows the efflux of Alimta® out of the cell (17). These molecules involved in metabolism of Alimta® may also affect Alimta® treatment for NSCLC (18, 19).

However, the clinical utility of selecting patients for treatment with Alimta® based on expression of these Alimta®-associated molecules has yet to be verified. This study was designed to investigate the relationship in NSCLC between *in vitro* chemosensitivity to Alimta® and clinicopathological factors, as well as gene expression of Alimta®-associated molecules, as a means of clarifying the differences in chemosensitivity to Alimta® among histological cell types and to identify potential molecular markers for Alimta® treatment.

Materials and Methods

Patients and sample collection. In this study, we analyzed fresh surgical specimens from 69 patients with NSCLC who underwent surgical resection at Kyushu University Hospital from April 2009 to March 2011. None of the patients had received any treatment prior to surgery. Of the 69 specimens, 55 were AD and 14 were SCC. Informed consent was obtained for this study, and it was approved by the Ethical Committee for Clinical Research of Kyushu University (No. 23-148).

In vitro chemosensitivity assay. Chemosensitivity of the surgically resected NSCLC tissues to Alimta®, 5-fluorouracil (5-FU), and gemcitabine was examined. Immediately following surgery, fresh tumor specimens (6 mm^2) were placed in culture medium and sent for laboratory testing. The succinate dehydrogenase inhibition (SDI) test was conducted as previously described (20). Briefly, the tissue specimens were digested to obtain single-cell suspensions which were then incubated for 72 h with cancer therapeutic drugs. The SDI assay was performed to measure cell viability, which entailed addition of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (0.4 mg/ml) to the cells with different concentrations of sodium succinate, followed by measurement of absorbance at 540 nm using a microtiter plate (spectrophotometer). The SD activity was determined as the optical density per milligram of protein. Drug chemosensitivity was expressed as a percentage of the SD activity in drug-treated cells to that of the control cells. Chemosensitivity rates were calculated as: chemosensitivity rate (%)=(T−B)/(U−B) × 100% (T: absorbance determined when tumor cells were exposed to drugs; U: absorbance of untreated cells; B: absorbance when no drug or MTT was added). Samples exhibiting greater than the median chemosensitivity rate were defined as 'Resistant', and samples exhibiting less were labeled 'Sensitive'.

Real-time PCR (qPCR) of Alimta®-related molecules. mRNA expression levels of the six Alimta®-associated molecules (TYMS, DHFR, GARFT, FPGS, GGH, RFC) were evaluated by qPCR. Immediately after surgery, the specimens were placed in liquid nitrogen and stored at −80˚C until later use. Total RNA was extracted from each of the resected tumor specimens using ISOGEN and Ethachinmate (Nippon Gene, Tokyo, Japan). cDNA was synthesized from RNA using Super Script™ III First-Strand Synthesis Super Mix (Invitrogen, CA, USA) according to the manufacturer's instructions. qPCR was performed with commercial TaqMan® Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol except with optimized primer and probe concentrations (*TYMS*: Hs00426586_m1, *DHFR*: Hs00758822_s1, *GARFT*: Hs00894582_m1, *FPGS*: Hs00191956_m1, *GGH*: Hs00914163_m1, RFC: Hs00953344_m1). β-Actin was used as an internal control (*ACTB*: Hs01060665_g1), and human reference RNA (Promega, Madison, WI, USA) was used as a standard for quantitation (21).

Immunohistochemistry. We performed immunohistochemical staining for TYMS, GGH and RFC on tissue available for 42 out of the 69 cases. Tumor sections were assessed immunohistochemically using mouse monoclonal antibody to TYMS (clone TYMS106, 1:50; Dako, Tokyo, Japan), rabit polyclonal antibody to GGH (HPA025226, 1:500; Atlas Antibodies, Tokyo, Japan) and rabit polyclonal antibody to RFC (sc-98971, 1:50; Santa Cruz, Tokyo, Japan). Briefly, 4-μm sections were deparaffinized in xylene and dehydrated in an ethanolic series. For antigen retrieval, slides were immersed in 0.01 M sodium citrate buffer (pH 6.0) for GGH and RFC, or in Target Retrieval Solution, pH 9 (Dako) for TYMS, and autoclaved at 121˚C for 15 min. The sections were washed and immersed in 1.5% hydrogen peroxide and absolute methanol to deactivate endogenous peroxidases. After blocking nonspecific binding of antibodies, the specimens were incubated at room temperature with primary antibodies at 4˚C overnight. Histological signal was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB tablet 049-22831; WAKO, Tokyo Japan) according to the manufacturer's instructions. Two investigators, including one general pathologist, who were blinded to any information about the samples, evaluated the levels of expression.

For evaluation of TYMS and RFC protein expression, the staining intensity was graded on a scale of 0 to 3 (0, negative; 1, weak positive; 2, positive, 3, highly positive) (Figure 1A and C). The percentage of positively stained tumor cells was evaluated as a proportion score (0 to 100). The immunohistochemistry score was calculated by multiplying the staining intensity by the percentage of stained tumor cells (H score: 0 to 300) as described in previous studies (22, 23). The selection of a cut-off score for protein expression was based on receiver operating characteristic (ROC) curve analysis. The expression of GGH was determined to be positive when the proportion of tumor cells with strong cytoplasmic staining was 10% or more (Figure 1B) as described in a previous study (24).

Statistical analysis. The relationships between the clinicopathological factors and chemosensitivities were analyzed by the χ^2 test and Fisher's exact test. The correlations between the expressions of Alimta®-related molecules and chemosensitivity were evaluated using the nonparametric Wilcoxon test. The correlations were considered significant when $p<0.05$. All analyses were performed with JMP statistical software, version 9.0.2 (SAS, Tokyo, Japan).

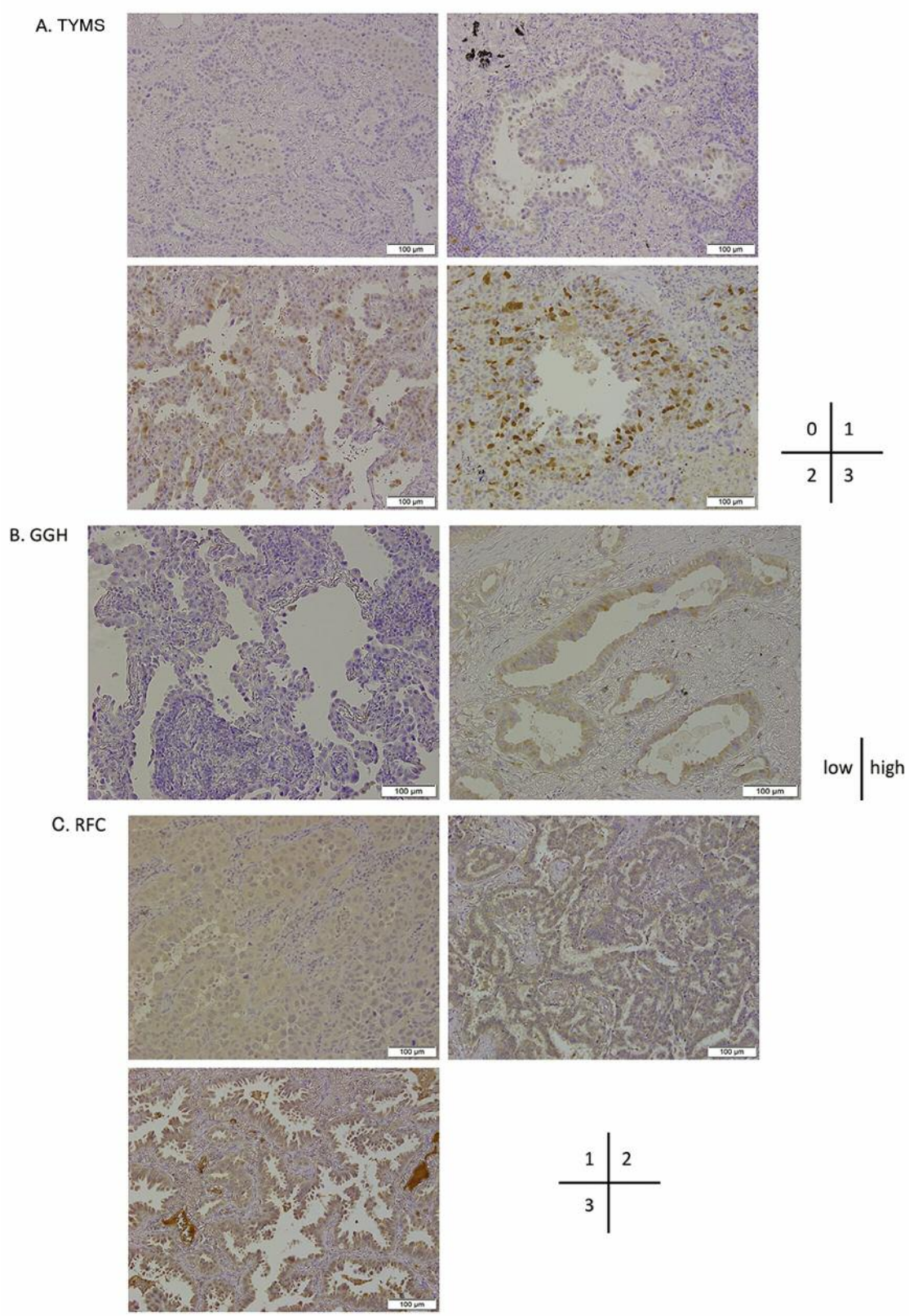

Figure 1. *Representitive sections for immunohistochemical scoring of Alimta®-related protein expression in primary lung tumors: Immunohistochemical staining of thymidylate synthase (TYMS) with indicated intensities (a), of gamma glutamyl hydrase (GGH) according to the cut-off level (b) and of reduced folate carrier (RFC) with indicated intensities (c).*

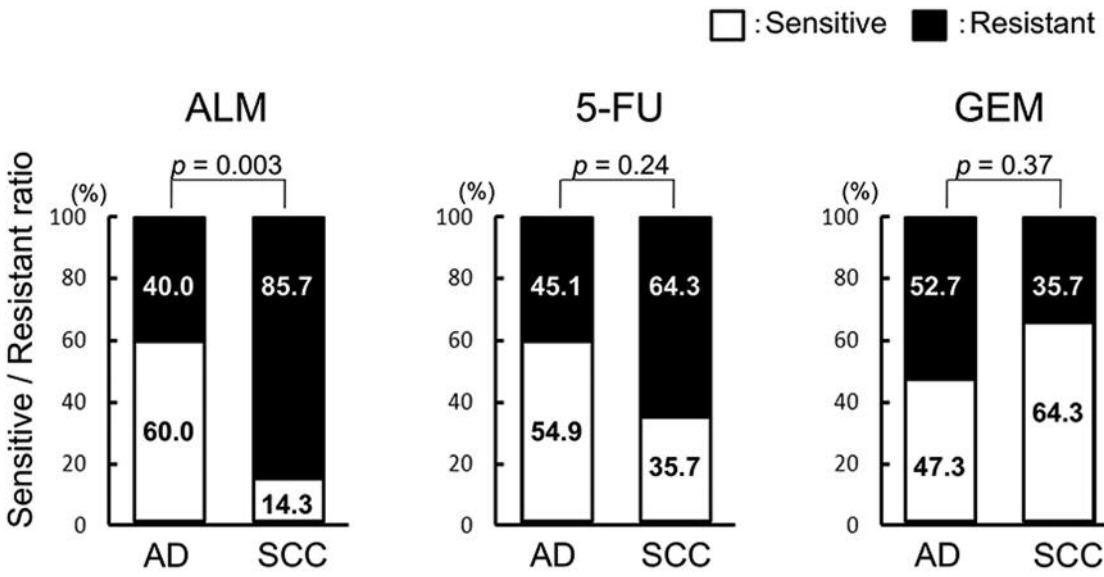

Figure 2. *The relationship between histological type and chemosensitivity to anti-metabolic drugs. Chemosensitivity in 69 samples: 55 adenocarcinomas (AC) and 14 squamous cell carcinomas (SCC). ALM: Pemetrexed (Alimta®); 5-FU: 5-fluorouracil; GEM: gemcitabine.*

Results

Chemosensitivity to anti-metabolic drugs according to histology. Firstly, we analyzed the association between histological type and *in vitro* chemosensitivity of three anti-metabolic agents (Alimta®, 5-FU, gemcitabine) using the SDI test. Among the three drugs, only chemosensitivity to Alimta® significantly differed between SCC and AD samples (p=0.003; Figure 2): 86% of SCC samples were resistant to Alimta®, while only 40% of AD samples were resistant to Alimta®.

Differences in expression of Alimta®-associated genes according to histology. We measured the transcript levels of the Alimta®-associated enzymes (TYMS, DHFR, GARFT, FPGS, and GGH) and the carrier molecule (RFC) by qPCR in AD and SCC clinical samples in order to analyze the relationship between gene expression and chemosensitivity to Alimta®. The chemosensitivity differences between AD and SCC in the Alimta® pathway were examined with respect to the correlation between histological type and transcript levels. TYMS expression was significantly elevated in SCC samples compared to AD samples (1.03 *vs*. 0.41, p=0.003; Table I), while RFC expression was significantly decreased in SCC samples compared to AD samples (0.16 *vs*. 0.90, p=0.03; Table I).

The relationship between chemosensitivity to Alimta® and clinicopathological factors in adenocarcinomas. Because 40% of the AD samples showed resistance to Alimta®, we analyzed the correlation between the chemosensitivity to Alimta® and clinicopathological factors (age, sex, smoking status, epidermal growth factor receptor (*EGFR*) mutation status) in AD samples, but no significant relationships were observed (Table II).

The differences in expression levels of Alimta®-associated genes in adenocarcinomas with respect to Alimta® chemoresistance. We analyzed the correlation between chemosensitivity to Alimta® and the transcript levels of the enzymes and the carrier molecule involved in Alimta® metabolism. *GGH* expression was significantly elevated in the chemoresistant AD samples relative to the chemosensitive samples (sensitive *vs*. resistant=0.16 *vs*. 0.39, p=0.03; Table III). No relationships between chemosensitivity to Alimta® and expression levels of the other five molecules were observed in AD.

The relation between mRNA expression and protein expression of Alimta®-associated genes. In ord or to ensure that mRNA expression of Alimta®-associated genes represent fuctional features in the tumors, we assessed the protein expression of TYMS, GGH and RFC by immunohistochemistry. Comparison of mRNA expression levels of these Alimta®-related genes according to the protein expression status showed that mRNA expression correlated well with the protein expression status in cancer cells (TYMS, GGH and RFC: p=0.0475, 0.0466 and 0.0410, respectively; Figure 3).

Discussion

Recent subset analyses of the three randomized phase III trials conducted for patients with advanced NSCLC revealed that patients with non-SCC treated with Alimta® had significantly

Table I. *Correlation between chemosensitivity Alimta® according to histology and expression of Alimta®-associated molecules.*

Alimta®-associated molecule		Transcript level		
		AD	SCC	*p*-Value
Target enzymes	*TYMS*	0.41±0.06	1.03±0.23	**0.003**
	DHFR	0.41±0.01	0.12±0.03	0.11
	GARFT	0.49±0.16	0.07±0.01	0.22
Carrier molecule	*RFC*	0.90±0.26	0.16±0.02	**0.03**
Metabolic enzymes	*FPGS*	1.07±0.23	0.58±0.11	0.53
	GGH	0.22±0.05	0.08±0.03	0.47

Data are the mean±standard error. AD: Adenocarcinoma, SCC: squamous cell carcinoma; *TYMS*: thymidylate synthase; *DHFR*: dihydrofolate reductase; *GARFT*: glycinamide ribonucleotide formyltransferase; *RFC*: reduced folate carrier; *FPGS*: folylpolyglutamate synthase; *GGH*: gamma glutamyl hydrase.

Table III. *Correlation between chemosensitivity to Alimta® and expression of Alimta®-associated molecules in adenocarcinomas.*

Alimta®-associated molecule		Transcript level		
		Sensitive	Resistant	*p*-Value
Target enzymes	*TYMS*	0.45±0.08	0.29±0.05	0.52
	DHFR	0.29±0.08	0.72±0.27	0.09
	GARFT	0.27±0.07	1.08±0.54	0.16
Carrier molecule	*RFC*	0.71±0.21	1.42±0.79	0.07
Metabolic enzymes	*FPGS*	1.12±0.30	0.94±0.23	0.66
	GGH	0.16±0.05	0.39±0.12	**0.03**

Transcript levels in 55 adenocarcinoma specimens. Data are the mean±standard error. *TYMS*: Thymidylate synthase; *DHFR*: dihydrofolate reductase; *GARFT*: glycinamide ribonucleotide formyltransferase; *RFC*: reduced folate carrier; *FPGS*: folylpolyglutamate synthase; *GGH*: gamma glutamyl hydrase.

Table II. *Correlation between chemosensitivity to Alimta® and clinicopathological factors in 55 adenocarcinoma samples.*

Factor (categories)	Sensitive (n=28)	Resistant (n=27)	*p*-Value
Age (>70 years/≥70 years)	15/13	7/20	0.06
Sex (Male/female)	11/17	17/10	0.11
Smoking (Ever/never)	13/15	20/7	0.06
EGFR mutation (Positive/negative)	16/12	15/12	0.91

EGFR: Epidermal growth factor receptor.

better survival than those given alternative treatments (hazard ratio=0.70 to 0.84) (2, 7, 8). These effects were not evident in the patients with SCC, and even worse results were shown among them. In these trials, there were differences in the settings for the treatment phases and in the drug combinations (9). To our knowledge, no direct evidence supporting histological differences in sensitivity to Alimta® has been demonstrated by *in vitro* chemosensitivity assays of cells. We hereby report the first evidence supporting histology-dependent differences in chemosensitivity to Alimta® by *in vitro* SDI tests using fresh tumor tissues (Figure 2). Our consistent results support the robustness of our SDI assay as a surrogate system for the analysis of chemosensitivity *in vivo*.

The present results demonstrated differences in the expression levels of Alimta®-associated genes according to histology. The *TYMS* mRNA expression was higher and the *RFC* mRNA expression lower in SCC than in AD (p=0.003 and p=0.03, respectively). The immunohistochemical study for TYMS, GGH and RFC confirmed that the mRNA expression level correlated with the protein level in cancer cells. Recently, the relationship between TYMS expression and sensitivity to Alimta® has been well investigated; however, only a few studies showed significant differences in TYMS expression between AD and SCC in patients with primary lung cancer. One study using 56 surgical specimens demonstrated that TYMS expression, at both the mRNA and protein levels, was higher in SCC than in AD (15). A large-scale study with Japanese patients (n=2150) also showed higher TYMS expression in SCC than in AD (24). The different TYMS expression levels observed between the two cell types may partially account for the observed differences in chemosensitivity to Alimta®; however, further studies are needed to clarify this relationship.

Polymorphisms in the *SLC19A1* gene that encodes RFC were shown to be associated with differences in the survival of patients with NSCLC receiving Alimta®-based chemotherapy (19). Another study showed that the SLC19A1 expression levels in pemetrexed-resistant small cell lung cancer cell lines were significantly decreased relative to those of parental cells (25). However, there have so far been no reports demonstrating differences in the RFC expression level among histological cell types. Our data provide the first indication that lower RFC expression might be correlated with chemoresistance to Alimta® in patients with SCC of the lung (Table I).

In regard to factors associated with chemosensitivity of lung adenocarcinomas to Alimta®, recent reports showed that low TYMS expression was correlated with the efficacy of Alimta®-based chemotherapy in patients with AD (11, 26). Chen *et al*. reported that patients with AD with low TYMS protein levels had a longer progression-free survival and a longer overall survival in the second or subsequent lines of Alimta® monotherapy, relative to patients with AD with high TYMS expression (11). In cell lines, it was reported that Alimta®-resistant adenocarcinoma clonal sublines expressed more TYMS than the parental cells from which they were derived (27).

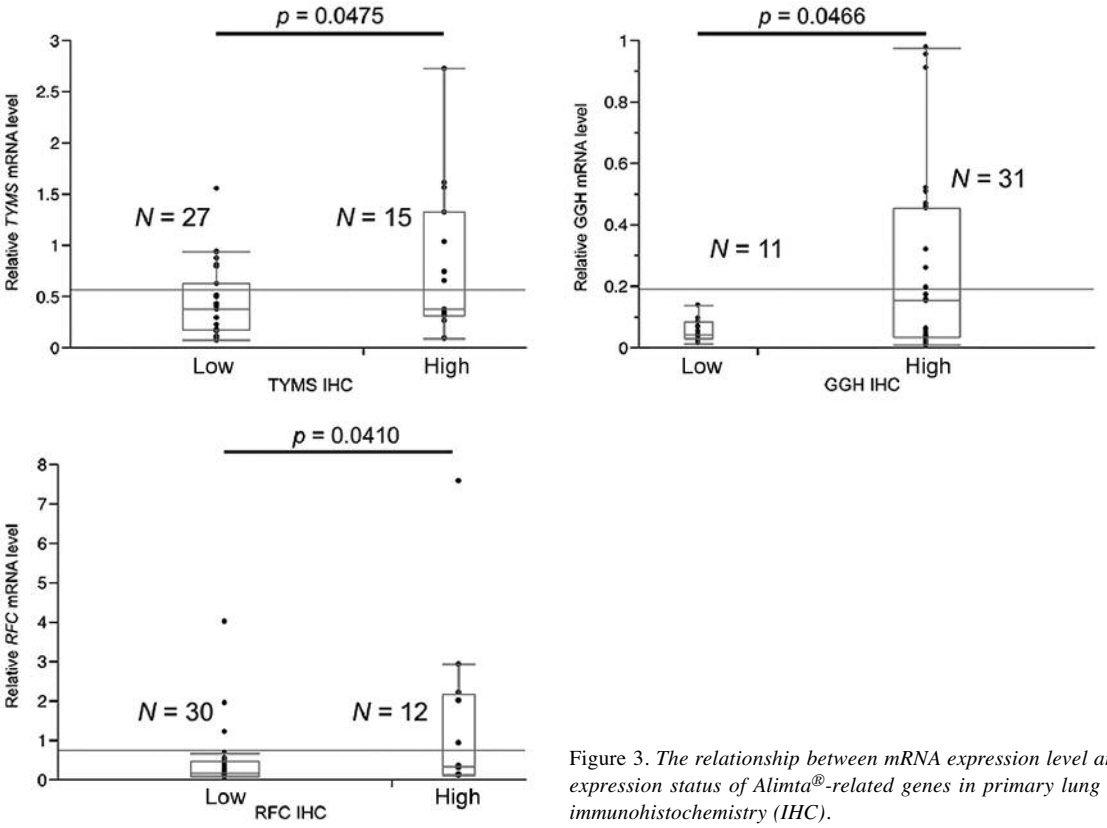

Figure 3. *The relationship between mRNA expression level and protein expression status of Alimta®-related genes in primary lung cancer by immunohistochemistry (IHC).*

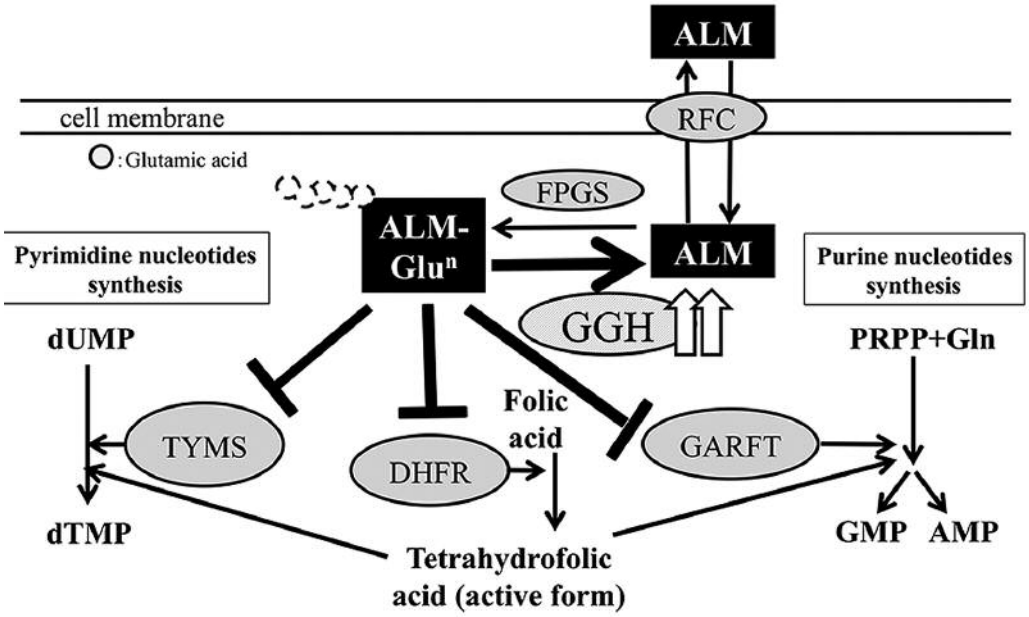

Figure 4. *Hypothetical model for the mechanisms of chemoresistance to Alimta® (ALM: pemetrexed) in adenocarcinomas. Chemoresistance to Alimta® in this cell type might be caused by high gamma glutamyl hydrase (GGH) expression. ALM-Glu^n: Pemetrexed converted to polyglutamate forms; TYMS: thymidylate synthase; DHFR: dihydrofolate reductase; GARFT: glycinamide ribonucleotide formyltransferase; RFC: reduced folate carrier; FPGS: folylpolyglutamate synthase; dUMP: deoxyuracilmonophosphate; dTMP: deoxythyminemonophosphate; PRPP: phosphoribosyl pyrophosphate; GMP: guanosine monophosphate; AMP: adenosine monophosphate.*

On the other hand, there have been a few investigations into the relationship between GGH and chemosensitivity to Alimta®. In a pharmacogenetic study involving clinical trials (28), polymorphisms in the *GGH* gene correlated with chemotoxicity and OS; however, the relationship between GGH expression and Alimta® chemosensitivity remains obscure. In our data, higher *GGH* expression was observed in the Alimta®-resistant AD samples compared to the Alimta®-sensitive samples (Table III). Furthermore, of the 20 AD samples that exhibited Alimta® sensitivity despite high TYMS expression, when the expression was dichotomized at the median level, 18 samples exhibited low *GGH* expression (90.0%). Among the eight samples that showed Alimta® resistance despite low TYMS expression, seven showed high *GGH* expression (87.5%). However, 23 out of the 25 samples displaying Alimta® sensitivity despite low RFC expression had low *GGH* expression (92.0%), while eight out of the 12 samples that showed Alimta® resistance despite high *RFC* expression had high *GGH* expression (66.7%). These results suggest that TYMS, RFC and GGH may independently correlate with chemosensitivity to Alimta®. Since TYMS expression varied considerably among individuals – for example, certain SCCs expressed lower TYMS than did ADs (24) – it is difficult to claim that TYMS expression alone affects chemosensitivity to Alimta®. These data imply that multiple genes influence chemosensitivity to Alimta®. A possible model of adenocarcinoma is depicted in Figure 4.

The main limitation of this study was the ambiguity of whether these gene expression differences are a cause or an effect of the chemosensitivity to Alimta®. In order to examine the accuracy of these hypotheses-generating findings, experiments in NSCLC cell lines would be necessary. Further studies investigating protein levels and the catalytic activities of these Alimta®-associated enzymes may help more accurately select patients that will benefit from treatment. Finally, the findings from our small-scale study should be examined in a prospective study with larger patient numbers.

This study suggests that the histology-dependent differences in *TYMS* and *RFC* gene expression levels are attributed to the histology-dependent differences in chemosensitivity to Alimta®. In ADs, *GGH* gene expression was associated with chemosensitivity to Alimta®. A clear correlation detected between expression of the Alimta®-associated genes and chemosensitivity to Alimta® would benefit the individualization of NSCLC therapy. Furthermore, it could help patients for whom Alimta® treatment is not fully effective by allowing selectively targeting the molecules associated with Alimta® resistance in these cases.

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