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In vitro sensitivity to platinum-derived drugs is associated with expression of thymidylate synthase and dihydropyrimidine dehydrogenase in human lung cancer

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Abstract. Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) are critical enzymes in nucleic acid metabolism. Proliferating cell nuclear antigen (PCNA) is a specific protein that is correlated with proliferative activity of cells. The TS gene has a variable number of tandem repeats (VNTR) in its 5'-untranslated region and a single nucleotide polymorphism (SNP) in the VNTR area. We examined the association of *in vitro* sensitivity to anticancer drugs with TS polymorphism, TS, DPD, and PCNA mRNA expression using human lung cancer tissues. Seventy-eight surgically resected lung cancer tissues were tested for *in vitro* sensitivity to 5-fluorouracil, cisplatin (CDDP), carboplatin (CBDCA), irinotecan, docetaxel, and gemcitabine by histoculture and MTT assay. The TS polymorphisms were analyzed by PCR and PCR-RFLP. TS, DPD, and PCNA mRNA expression levels were quantified by real-time RT-PCR and normalized relative to ß-actin mRNA expression. The inhibition rates (IRs) of CDDP and CBDCA were significantly correlated with TS/PCNA, the ratio of TS/actin and PCNA/actin, and DPD/PCNA, the ratio of DPD/actin and PCNA/actin. This correlation was further explored by subgroup analyses according to TS VNTR or TS functional type, in which 2R/3G, 3C/3G, or 3G/3G were classified into H-type group and 2R/2R, 2R/3C, or 3C/3C into L-type group. The associations of TS/PCNA and DPD/PCNA with the IRs of CDDP, CBDCA remained significant in the 3R/3R group and H-type group. These results suggest that *in vitro* sensitivity to platinum-derived drugs, CDDP and CBDCA, is associated with PCNA-normalized mRNA expression of TS and DPD in human lung cancer tissues, as affected by the TS polymorphism. The clinical significance of these pharmacogenomic markers for chemotherapy regimens with platinum-derived drugs should be investigated further for personalized treatment of lung cancer.

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Introduction

Lung cancer is the leading cause of death from cancer worldwide (1). Despite radical surgery therapy, the cumulative 5-year survival rate of patients with lung cancer is 57-79% even for stage I disease (2,3). Therefore, effective adjuvant chemotherapy is necessary in the treatment of lung cancer. Recent clinical trials suggested that post-operative adjuvant chemotherapy confers a survival benefit (4,5), and this is becoming the standard strategy for the treatment of lung cancer. One of the problems associated with adjuvant chemotherapy is that long-term survival is the only marker of the effectiveness, which means that all patients undergo therapy with the full duration of the protocol as long as it is tolerable. Thus, some of the patients receive onerous treatment in vain, which has an adverse effect on the quality of their life. To avoid ineffective treatment, it is important to predict the tumor's sensitivity to anticancer drugs.

Thymidylate synthase (TS) catalyzes the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate to form dTMP and dihydrofolate. TS is an important target for cancer chemotherapy because of its central, rate-limiting role in the *de novo* synthesis of dTTP (6). There have been a number of reports that TS expression is a predictive factor of the outcome of 5-fluorouracil (5-FU)-based chemotherapy (7,8). The TS gene is known to have variable number of tandem repeats (VNTR) satellite sequences in its 5'-untranslated region (5'-UTR) (9), and this polymorphism is associated with TS protein expression (10,11). Several clinical studies have indicated the association between TS VNTR genotype and the effectiveness of 5-FU-based chemotherapy (12,13). More recently, a single nucleotide polymorphism (SNP) was identified in the VNTR area, and this was suggested to be a potential predictive factor for the effectiveness of chemotherapy (14). In addition to TS, dihydropyrimidine dehydrogenase (DPD) expression in cancer tissue has been analyzed extensively as a predictive marker of the effectiveness of 5-FU-

based chemotherapy. DPD is the initial and rate-limiting enzyme in the chain of reactions in the catabolism of 5-FU. Accordingly, it has been suggested that high DPD activity is linked with a decrease in level of FdUMP, the active metabolite of 5-FU, resulting in a reduced effectiveness of the drug. Indeed, it has been reported that the DPD activity in tumor tissue is associated with sensitivity to 5-FU (8). These pharmacogenomic factors, TS expression, TS polymorphism, and DPD expression, have been analyzed mainly in gastrointestinal tumors and have attracted less attention in lung cancer because 5-FU was considered ineffective in this disease. However, recent clinical studies of UFT (tegafur and uracil) demonstrated that fluoropyrimidine is effective in lung cancer in an adjuvant setting (15-17), attracting more interest in TS and DPD as predictive factors.

Proliferating cell nuclear antigen (PCNA) is the specific protein that is correlated with the proliferative activity of cells. Several studies have suggested that PCNA expression may be an indicator of malignant potential in tumors because of its associations with the depth of invasion, organ metastasis, tumor differentiation, and tumor stage (18,19). Therefore, PCNA expression is an independent prognostic factor in cancer patients and the combination of TS, DPD, and PCNA expression may be useful predictive factors in cancer chemotherapy.

Cisplatin, a key drug in the chemotherapy of lung cancer, exerts its anticancer activity through Pt-DNA adduct formation that inhibits DNA synthesis (20). High repair activity of the Pt-DNA adduct is a major mechanism of drug resistance to cisplatin (21). TS and DPD are key enzymes in nucleotide metabolism, which is essential for DNA repair. This reaction is critical as it maintains the essential metabolic requirements for cell proliferation and growth. Together, TS, DPD, and PCNA may be predictive factors of the efficacy of cisplatin as well as fluoropyrimidine. Furthermore, many other anticancer drugs target DNA synthesis, suggesting that TS, DPD, and PCNA may also be useful predictors of the efficacy of multiple anticancer agents used in lung cancer therapy.

In the present study, we studied TS polymorphism, TS, DPD, and PCNA mRNA expression, and *in vitro* sensitivity to various anticancer drugs using surgically resected non-small cell lung cancer specimens. The pharmacogenomic factors were evaluated by comparison with the results of *in vitro* sensitivity testing with 5-FU, cisplatin, carboplatin, irinotecan, docetaxel, and gemcitabine.

Materials and methods

Samples. A total of 78 cancer tissues were obtained surgically from 78 patients with primary non-small cell lung cancer (NSCLC), all of whom had given their written informed consent. All patients were Japanese and were comprised of 56 men and 22 women, ranging in age from 44 to 86 years, with a mean age of 66.0 years. Parts of the surgically obtained tissues were used for nucleic acid isolation and *in vitro* histoculture chemosensitivity testing with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. The remaining part of each sample was fixed with formalin and used for further histological examination to confirm the diagnosis postoperatively. All of the histological examinations were performed after staining with hematoxylin and eosin (H&E). Ethical approval for the project was obtained from the Kanazawa University School of Medicine Ethics Committee.

Histoculture with anticancer drugs and the MTT assay. RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin was prepared as complete medium. Samples of about 1 g of the tumor tissues were washed in complete medium, followed by cutting into pieces of 1-2 mm in diameter as soon as possible. The pieces of the tumor tissue were then placed on collagen sponge gel manufactured from pig skin (Spongostan®; Health Design Industries, Rochester, NY) and cultured in complete medium. Six anticancer agents, 5-fluorouracil (5-FU), cisplatin (CDDP), carboplatin (CBDCA), irinotecan (SN-38), docetaxel (DOC), and gemcitabine (GEM), were added to the culture medium. The concentrations of the anticancer agents were ten times the clinically achievable peak plasma concentrations (PPCs) in man calculated based on LD_{50} values in mice, which were defined as (22): $\log (PPC) = -0.788 + [0.755 \times \log (LD_{50})]$.

After histoculture for 7 days, a modification of the MTT assay described by Mosmann (23) was performed. Briefly, aliquots of 40 μl of MTT solution prepared by dissolving solid MTT (2.0 mg/ml) and 0.25% collagenase in phosphate-buffered saline (PBS) were added to each culture well and incubated at 37˚C for 4 h. The MTT-formazan products were dissolved in 0.3 ml of DMSO. Then, the solutions were transferred to 96-well microplates and the absorbance was read at 540 nm. The mean absorbance from triplicate assays was used to calculate the inhibition rate (IR) as follows (24,25):

$$\text{IR (\%)} = \left[1 - \frac{\text{Absorbance of the treated tumor / weight of the treated tumor (g)}}{\text{Absorbance of the control tumor / weight of the control tumor (g)}}\right] \times 100$$

In cases where the result calculated using this formula was less than 0%, the IR was considered to be 0%.

Nucleic acid isolation. Specimens of about 1 g of the tissues were stored immediately at -80˚C until DNA and RNA isolation. Genomic DNA was isolated using a QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer's. Total RNA was isolated by the single-step guanidinium isothiocyanate method (26).

Analysis of TS genotypes. For analysis of the TS VNTR and G/C SNP in the 5'-UTR, PCR and PCR-RFLP methods were used as described previously (10,14). Briefly, a fragment containing the repeats was amplified using the primers TS 25: 5'-AGGCGCGCGGAAGGGGTCCT-3' and TS 18: 5'-TCCGAGCCGGCCACAGGCAT-3'. Aliquots of amplified fragments were separated on 3% agarose gels and the TS VNTR genotype was determined. Samples showing the 2R/3R or 3R/3R genotypes were further analyzed for the G/C SNP by PCR-RFLP. *Hae*III digestion of the 3R fragment produced bands of 66, 37, 28, and 10 bp for the 3G allele, and 94, 37 and 10 bp for the 3C allele after separation on 3% agarose gels. Accordingly, TS genotype was classified as

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Table I. Genotype frequency of TS polymorphism in NSCLC.

	2R/2R	2R/3R		3R/3R			3R/5R	Total
		2R/3G	2R/3C	3G/3G	3G/3C	3C/3C		
Total	2	13	15	14	21	12	1	78
Gender								
Male	1	11	10	12	15	7		56
Female	1	2	5	2	6	5	1	22
Histological type								
Adeno	1	11	9	8	14	8	1	52
Squamous cell	1	2	5	6	15	3		22
Others[a]			1		2	1		4

[a]Others contain adenosquamous, large cell, and mucoepidermoid carcinoma.

2R/2R, 2R/3G, 2R/3C, 3G/3G, 3G/3C, or 3C/3C based on the genotype of the VNTR and the SNP. Analysis was performed at least twice to confirm the genotype.

Quantification of TS, DPD, and PCNA mRNA. Quantification of the mRNA levels was carried out using a real-time fluorescence detection method as described previously (27). The quantities of TS, DPD, and PCNA mRNA were expressed as ratios relative to that of ß-actin mRNA. The primer and probe sequences for TS and ß-actin were listed previously (11). Those for DPD and PCNA were as follows: i) DPD: forward primer, GCAGACTC GAGACTGTAGGCACT; reverse primer, ACTCTCGATGTC CGCCGA; probe, FAM-CCATGGCCCCTGTGCTCAGTA AGG-TAMRA; ii) PCNA: forward primer, GTGCAAAAGAC GGAGTGAAATTT; reverse primer, ATCGACATTACTTG TCTGTGACAATTTA; probe, FAM-TGTTTCCATTTCCAA GTTCTCCACTTGCAG-TAMRA.

Statistical analysis. Groups were compared by the χ^2 test and the Mann-Whitney U test. The correlations between TS, DPD, and PCNA mRNA expression and *in vitro* chemosensitivity were analyzed by Pearson's correlation coefficient test. $p<0.05$ was considered to indicate significance.

Results

Frequencies of TS VNTR and G/C SNP in NSCLC. The genotype frequencies of TS polymorphisms are summarized in Table I. TS genotypes including repeat sequences longer than 3 were excluded from further analysis in this study because of their infrequency. There were no significant associations between the clinicopathological features, including patient's gender, age, and histological type, and genotype frequency of TS polymorphism.

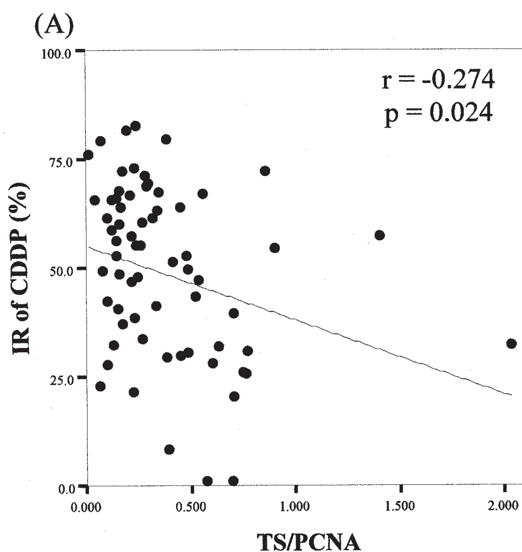
TS, DPD, and PCNA mRNA expression in NSCLC. We quantified TS, DPD, and PCNA mRNA levels in 77 available samples from the 78 lung cancer tissues using a real-time fluorescence detection method. The histological type of the tumor was associated with DPD and PCNA mRNA expression.

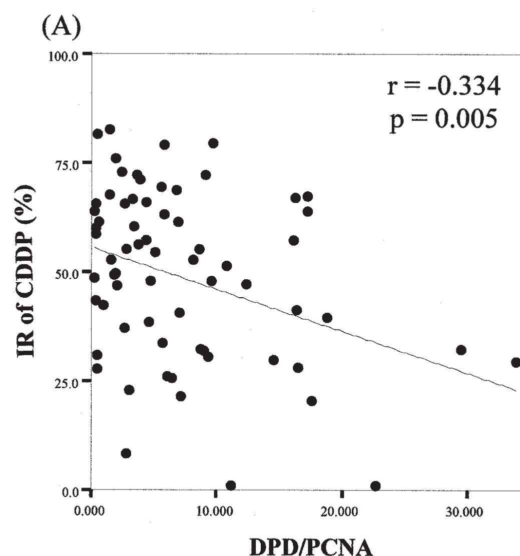
Table II. TS, DPD, and PCNA mRNA expression in NSCLC.

	No.	TS/actin	DPD/actin	PCNA/actin
Total	77	0.184±0.210	2.807±3.227	0.704±1.114
Gender				
Male	55	0.186±0.199	2.371±2.202	0.743±1.283
Female	22	0.178±0.240	3.898±4.846	0.606±0.491
		p=0.423	p=0.143	p=0.761
Histological type				
Adeno	51	0.141±0.099	3.502±3.658	0.443±0.347
Squamous cell	22	0.300±0.338	1.546±1.484	1.389±1.870
		p=0.097	p=0.001	p<0.001

Adenocarcinoma tissues showed higher levels of DPD/actin than squamous cell carcinoma tissues, and lower levels of PCNA/actin (Table II). There were no significant associations of the other clinicopathological features with TS, DPD, or PCNA mRNA expression. There was no relationship between TS, DPD, and PCNA mRNA expression and the malignant potential in tumors, including the size of the tumor, tumor differentiation, pleural invasion, vascular invasion, lymph nodes metastasis, and tumor stage (data not shown). There were no relationships between TS genotype and TS, DPD, or PCNA mRNA expression (data not shown).

Association of in vitro sensitivity to anticancer drugs with TS, DPD, and PCNA mRNA expression. The inhibition rates (IRs) of anticancer drugs described in Materials and methods were obtained in 68 samples for CDDP, CBDCA, 5-FU, and SN-38, 65 samples for DOC, and 66 samples for GEM. There were no significant associations of the IRs of any anticancer drugs with clinicopathological features, TS polymorphism, or the mRNA expression of TS, DPD, or PCNA. Next, we calculated the ratio of TS/actin and PCNA/actin, expressed as TS/PCNA.





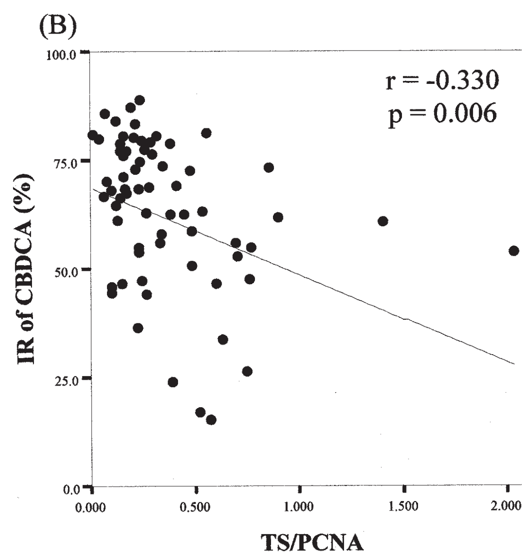


Figure 1. The level of TS/PCNA, the ratio of TS/actin and PCNA/actin, was correlated with the IRs of CDDP (A) and CBDCA (B) with correlation coefficients of -0.274 (p=0.024) and -0.330 (p=0.006), respectively.

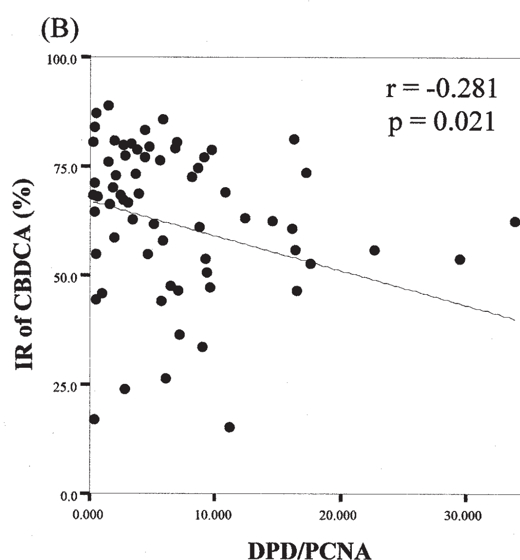


Figure 2. The level of DPD/PCNA, the ratio of DPD/actin and PCNA/actin, was correlated with the IRs of CDDP (A) and CBDCA (B) with correlation coefficients of -0.334 (p=0.005) and -0.281 (p=0.021), respectively.

This value represents the TS expression independent of cell cycle populations of analyzed cancer cells through normalization by G1 phase-specific gene expression of PCNA. DPD/PCNA, the ratio of DPD/actin and PCNA/actin, was also calculated and the PCNA-normalized values were compared with IRs. The results indicated that the IRs of CDDP and CBDCA were linked to TS/PCNA and DPD/PCNA. The level of TS/PCNA was significantly correlated with the IRs of CDDP and CBDCA, with correlation coefficients of -0.274 (p=0.024) and -0.330 (p=0.006), respectively (Fig. 1). The level of DPD/PCNA was also significantly correlated with the IRs of CDDP and CBDCA, with correlation coefficients of -0.334 (p=0.005) and -0.281 (p=0.021), respectively (Fig. 2). There were no relationships between the IRs of other anticancer drugs and PCNA-normalized TS or DPD mRNA expression.

Although TS polymorphism was not associated with IRs, it may affect the correlation between mRNA expression and IRs. Therefore, subgroup analyses were performed according to TS genotypes. The genotype was first classified by the TS VNTR. The results of subgroup analysis indicated that TS/PCNA was correlated with the IRs of CDDP, CBDCA, and SN-38 (p=0.019, 0.010, and 0.034, respectively) in the 3R/3R genotype group. DPD/PCNA was also correlated with the IR of CDDP (p=0.025) when considering 3R/3R genotype (Table III). There were no correlations of TS, DPD, or PCNA mRNA expression with the *in vitro* chemosensitivity in the 2R-containing genotype group (2R/2R and 2R/3R). We then stratified the TS genotypes by the combination of VNTR and SNP, in which 3G was considered a high expression allele and 2R or 3C as low expression alleles according to the results of *in vitro* functional analysis reported previously (14). Based on this consideration, TS genotypes of 2R/3G, 3C/3G, and 3G/3G were classified as high expression (H) type and 2R/2R, 2R/3C, or 3C/3C as low expression (L) type. In the H-type group, TS/PCNA was correlated with the IRs of CDDP and CBDCA (p=0.037 and 0.006, respectively),

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. Association of the level of TS/PCNA and DPD/ ith the IRs of CDDP, CBDCA, and SN-38 when considering the genotype of TS VNTR.

	Total (n=68)	2R/2R and 2R/3R (n=27)	3R/3R (n=40)
TS/PCNA			
CDDP	r=-0.274	r=-0.155	r=-0.370
	p=0.024	p=0.440	p=0.019
CBDCA	r=-0.330	r=-0.189	r=-0.404
	p=0.006	p=0.345	p=0.010
SN-38	r=-0.168	r=0.069	r=-0.336
	p=0.170	p=0.732	p=0.034
DPD/PCNA			
CDDP	r=-0.334	r=-0.311	r=-0.354
	p=0.005	p=0.114	p=0.025
CBDCA	r=-0.281	r=-0.305	r=-0.264
	p=0.021	p=0.122	p=0.099
SN-38	r=0.000	r=0.052	r=-0.014
	p=0.999	p=0.798	p=0.932

Table IV. Association of the level of TS/PCNA and DPD/PCNA with the IRs of CDDP, CBDCA, and SN-38 when considering the genotype of G/C SNP.

	Total (n=68)	H-type group (n=42)	L-type group (n=25)
TS/PCNA			
CDDP	r=-0.274	r=-0.323	r=-0.183
	p=0.024	p=0.037	p=0.380
CBDCA	r=-0.330	r=-0.420	r=-0.193
	p=0.006	p=0.006	p=0.355
SN-38	r=-0.168	r=-0.245	r=-0.037
	p=0.170	p=0.117	p=0.862
DPD/PCNA			
CDDP	r=-0.334	r=-0.316	r=-0.321
	p=0.005	p=0.041	p=0.118
CBDCA	r=-0.281	r=-0.269	r=-0.251
	p=0.021	p=0.085	p=0.226
SN-38	r=0.000	r=0.029	r=0.011
	p=0.999	p=0.857	p=0.959

and DPD/PCNA with the IR of CDDP (p=0.041) (Table IV). There were no correlations between TS, DPD, or PCNA mRNA expression with *in vitro* chemosensitivity in the L-type group.

Discussion

In the present study, we investigated the associations between *in vitro* sensitivity to anticancer drugs and TS polymorphism and TS, DPD, and PCNA mRNA expression in NSCLC to explore pharmacogenomic markers. The results indicated that there were no correlations between the IRs of any anticancer drugs and the TS polymorphism or TS, DPD, or PCNA mRNA expression. However, we observed associations between the IRs of platinum-derived drugs with the TS/PCNA, ratio of TS/actin and PCNA/actin, and with the DPD/PCNA, ratio of DPD/actin and PCNA/actin. Both TS/PCNA and DPD/PCNA were significantly correlated with the IRs of CDDP and CBDCA. It is uncertain why PCNA-normalized TS and DPD mRNA expression were associated with the efficacy of platinum-derived drugs. One possibility is that TS/PCNA and DPD/PCNA may represent the activity of nucleotide supply independent of cell cycle phase. TS and DPD are known to be expressed in a cell cycle-dependent manner, with higher levels of expression in S-phase (28). Therefore, TS and DPD mRNA expression increase with the size of the cell population in S-phase, which may conceal the roles of TS and DPD as indicators of nucleotide supply for DNA repair. On the other hand, PCNA-normalized mRNA expression levels can be used as cell cycle-independent markers because PCNA mRNA is expressed specifically at S-phase of the cell cycle. Taken together, these observations suggest that TS/PCNA and DPD/PCNA may be novel markers of the DNA repair capacity and thus may be linked with chemosensitivity to CDDP and CBDCA in lung cancer. This hypothesis should be tested further in large-scale clinical studies.

A number of clinical studies have indicated that TS and DPD expression can be used as novel predictive factors of the efficacy of 5-FU-based chemotherapy (7,8). Therefore, we expected that TS or DPD mRNA expression would be useful as a marker of chemosensitivity to 5-FU in lung cancer. However, the results of the present study revealed no associations between chemosensitivity to 5-FU and TS or DPD mRNA expression. This result is not surprising because lung cancer is biologically distinct from gastrointestinal tumors, which were the subjects of previous studies indicting the pharmacogenomic significance of TS and DPD. However, 5-FU is generally ineffective in lung cancer (29), but effective in gastrointestinal cancer. There may be unknown resistance markers of 5-FU in lung cancer that are more important than TS and DPD for predicting chemosensitivity to 5-FU. Although we found no significant roles of TS or DPD expression as pharmacogenomic markers of 5-FU sensitivity, our results do not exclude further exploration of these markers in lung cancer. Current results of chemosensitivity testing are based on an *in vitro* culture system and MTT assay. It is well known that not all *in vitro* chemosensitivity data reflect the clinical response and that *in vitro* culture conditions of tumor cells can markedly influence the data. Higashiyama *et al* reported the relationships between TS and DPD activities in NSCLC with *in vitro* sensitivity to 5-FU (30). The inconsistency in their results with those of the present study may be attributed to differences in culture conditions and/or assay methods used to obtain chemosensitivity data. Fluoropyrimidine has been validated to be effective in lung cancer in an adjuvant setting (15-17). TS and DPD expression, as well as other pharmacogenomic factors, should be investigated further in the area of fluoropyrimidine therapy for lung cancer.

Although there was no correlation between TS polymorphism and sensitivity to anticancer drugs, correlations

between the levels of TS/PCNA or DPD/PCNA and sensitivity to platinum-derived drugs were present in specific subgroups of TS polymorphism-i.e., the 3R/3R or H-type group. Tumors in either group were suggested to have higher translational activity of TS mRNA than the corresponding 2R-containing or L-type group. The correlation between TS expression and chemosensitivity may be dependent on the TS gene expression activity. Our previous study using gastric cancer cell lines showed similar effects of TS polymorphism. In this previous study, 3R/3R and 2R-containing groups showed different correlation curves between TS protein expression and sensitivity to 5-fluoro-5'-deoxyuridine (31). These results suggest that TS polymorphism and TS mRNA expression may be independent predictive factors of chemotherapy and should be analyzed in combination to improve the effectiveness of prediction.

With regard to the association between clinicopathological features and TS, DPD, and PCNA mRNA expression, the results indicated that adenocarcinoma tissues had higher levels of DPD/actin and lower levels of PCNA/actin than squamous cell carcinoma tissues. There were no relationships between TS, DPD, and PCNA mRNA expression and other clinicopathological features. Huang *et al* reported that squamous cell carcinoma showed higher levels of TS expression than adenocarcinoma detected immunohistochemically, while there was no significant difference in DPD expression (32). Higashiyama *et al* reported results similar to those described by Huang *et al*, although their results did not reach statistical significance (30). These reports and our results suggest that TS, DPD, and PCNA mRNA expression may have different ranges between adenocarcinoma and squamous cell carcinoma in lung cancer. This difference can affect the results of studies exploring pharmacogenomic markers in lung cancer. Therefore, samples homogeneous in histological type may need to be examined in future pharmacogenomic studies in lung cancer.

In conclusion, we observed the association between *in vitro* sensitivity to platinum-derived drugs with PCNA-normalized TS and DPD mRNA expression in NSCLC. These associations were affected by the TS polymorphism. These results warrant further large-scale clinical studies to clarify the role of TS genotyping and quantification of TS, DPD, and PCNA mRNA expression for prediction of the efficacy of chemotherapy in NSCLC.

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