Thymidylate synthase, dihydropyrimidine dehydrogenase, orotate phosphoribosyltransferase mRNA and protein expression levels in solid tumors in large scale population analysis

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Abstract. It has been reported that the expression of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and orotate phosphoribosyltransferase (OPRT) may predict the clinical efficacy of 5-fluorouracil (5-FU)-based therapy in cancer patients. We investigated the differences in the mRNA and protein expression of these enzymes in various tumor tissues. A total of 17,613 specimens of head and neck, gastric, colorectal, breast, lung and pancreatic cancer were collected from multiple facilities in Japan, and the mRNA and protein expression levels of the above enzymes were examined in 4,830 and 12,783 of these specimens, respectively. The mRNA levels were analyzed using RT-PCR in laser-captured microdissected formalin-fixed paraffin-embedded specimens, while the protein levels were analyzed by enzyme-linked immunosorbent assays. The median values of the relative TS, DPD and OPRT mRNA levels were 2.06, 0.803 and 1.17, respectively, while the median protein levels were 22.1, 134.8 and 3.81 ng enzyme/mg protein, respectively. The carcinomas were classified into two sets of four groups each using the overall median levels of TS and DPD or TS and OPRT as cutoff values. Approximately 60% of the gastric cancers exhibited elevated mRNA and protein expression levels of DPD, while >65% of the colorectal cancers showed low levels of DPD expression. Overall, 75% of the head and neck cancers exhibited high expression levels of DPD. Among the lung and pancreatic cancers, 50-74% showed low TS/high DPD expression. In conclusion, the mRNA expression and protein levels of TS, DPD and OPRT differed according to the type of cancer. The results of this large-scale population analysis are expected to be useful as reference data for predicting the relationship between the respective enzyme levels and the efficacy of 5-FU-based chemotherapy.

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Introduction

5-Fluorouracil (5-FU) is a common and widely used chemotherapy for the systemic treatment of gastrointestinal tract, breast and head and neck cancers. Since 5-FU is catabolized to fluoro-5,6-dihydrouracil (FUH_2) by dihydropyrimidine dehydrogenase (DPD), the initial and rate-limiting enzyme of pyrimidine catabolism, 5-FU has only limited efficacy and elicits a response of short duration. To improve the clinical response to 5-FU treatment, the optimal administration schedules and combination of 5-FU with other drugs or biochemical modulators have been investigated, and increase in the antitumor activity and decrease in patient toxicity of 5-FU have been obtained (1). However, a major drawback in the clinical use of 5-FU is the development of 5-FU resistance and the existence of natural resistance to the drug in some patients.

More than 80% of administered 5-FU is catabolized by DPD, an enzyme that has been detected in a variety of tissues (2,3). Human tumor xenografts with low expression levels of DPD mRNA and/or low DPD activity have been shown to respond better to 5-FU than tumors with high levels of DPD mRNA and enzyme activity (4). DPD activity has also been shown to be higher in the liver than in other tissues, but human tumor cells also exhibit considerable DPD activity (5). Many reports have discussed the relationship between DPD expression in tumor and the efficacy of 5-FU-based chemotherapy, but conflicting results have been obtained in clinical studies (2,6-9).

Thymidylate synthase (TS) enables reductive methylation of deoxyuridine monophosphate (dUMP), using 5,10-

methylentetrahydrofolate as a one-carbon donor, to deoxythymidine monophosphate (dTMP), and is the rate-limiting enzyme in the *de novo* biosynthesis of deoxythymidine triphosphate (dTTP), an enzyme essential for DNA replication. TS is inhibited by 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) derived from 5-FU, leading to the inhibition of DNA synthesis (10-12). A low rate of FdUMP formation and high TS expression level may explain, at least in part, the low efficacy of 5-FU in colorectal cancer patients receiving the drug by intravenous bolus administration (13,14). Several clinical studies have demonstrated the existence of a relationship between the TS expression in tumors and the clinical response and survival of cancer patients receiving 5-FU-based chemotherapy (6-9,15-17).

Orotate phosphoribosyltransferase (OPRT) is the main enzyme responsible for the phosphorylation of 5-FU in human cancer cells (18). Recently, several reports of basic and clinical research on the relationship between OPRT and the 5-FU phosphorylation pathway have been published (19-21). Furthermore, several clinical reports have demonstrated a relationship between the expression or activities of TS, DPD and OPRT in the tumors and the clinical response or survival of cancer patients receiving 5-FU-based chemotherapy (22,23).

Although the expression levels of the above-mentioned enzymes appear to be important predictors of the clinical response to 5-FU-based chemotherapy, no large-scale studies investigating the expression levels of these enzymes in clinically resected tumors have been reported until now. Previously, Fukushima and colleagues reported the TS and DPD activities in a total of 2,590 surgically resected gastric, colorectal, breast, and non-small cell lung cancers (24). We had conducted a large-scale population study to examine the intratumoral TS, DPD and OPRT mRNA and protein expression levels in head and neck and pancreatic cancers, in addition to the previously reported study on the expression of these enzymes in gastric, colorectal, breast and lung cancers. In regard to the expression levels of pyrimidine-metabolizing enzymes, while relatively large-scale population studies of the DPD activity in peripheral blood mononuclear cells (PBMC) have been reported (25,26), no large-scale studies have been conducted to examine the expression levels in solid tumors. There are several reports of small-scale studies on the mRNA expression levels of TS, DPD and OPRT, for example, in 40 breast cancers (27) and 37 colorectal cancers (23), and of the protein expression levels of these enzymes, for example, in 52 breast cancers (28) and 75 gastric cancers (21). However, conflicting results have been reported from these small-scale clinical studies with regard to the prognostic value of the DPD mRNA and protein expression levels for predicting the responses and outcomes of patients receiving 5-FU-based chemotherapy. There are no reference data for these enzyme expression levels in solid tumors.

In the present study, we measured the mRNA and protein expression levels of TS, DPD and OPRT in 17,613 surgically resected solid tumors collected from collaborating universities or hospitals in Japan. The aim of this study was to establish reference data for the expression levels of these enzymes by cancer type in the Japanese population, based on a large-scale analysis of specimens.

Materials and methods

Patients. A total of 17,613 surgical specimens were obtained from patients treated at 251 university or public hospitals in Japan; all the patients had provided written informed consent for the use of the specimens for this study. None of the patients had received any chemotherapy prior to the surgery. Tumor tissues not including the stroma were collected from the surgically resected specimens and used for the measurements of the mRNA and protein expression levels of TS, DPD and OPRT. Surgically resected non-cancerous tissue samples and metastatic lymph nodes were also obtained from the patients, where possible. The analyzed specimens are summarized according to the cancer type in Table I. Formalin-fixed, paraffin-embedded (FFPE) tumor specimens were used for the mRNA measurements, and fresh frozen specimens (stored at -80˚C until use) were used for the measurements of the protein expression.

Analysis of mRNA expression. A pathologist selected representative FFPE tumor specimens after examining hematoxylin and eosin-stained slides. Sections (10 μm) were stained with neutral fast red to enable histological visualization during laser-capture microdissection, which was performed to ensure that only the tumor cells were collected. In brief, RNA was isolated from the FFPE specimens using a novel proprietary procedure (Response Genetics, Los Angeles, CA; United States Patent Number 6,248,535). After the RNA isolation, cDNA was derived from each sample according to a previously described procedure. The target cDNA sequences were amplified using quantitative PCR and a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems, Foster City, CA]. The PCR reaction mixture used contained primers, dATP, dCTP, dGTP, dUTP, $MgCl_2$ and TaqMan buffer (all reagents were supplied by Applied Biosystems). The PCR conditions were 50˚C for 10 sec and 95˚C for 10 min, followed by 42 cycles at 95˚C for 15 sec and 60˚C for 1 min. The mRNA expression levels were expressed as values relative to those of ß-actin (ACTB) used as the internal reference (29-31).

Analysis of protein expression. The surgically resected specimens were homogenized with four volumes of 20-mM TBS buffer (pH 7.5) containing 0.1% (v/v) Tween-20 and centrifuged at 105,000 x g for 60 min. The supernatants were then used for the TS-ELISA, DPD-ELISA and OPRT-ELISA assays. Crude enzyme extracts were diluted with 20-mM TBS buffer (pH 7.5) containing 0.1% Tween-20 to determine the amount of TS, or with 20-mM PBS buffer (pH 7.0) containing 2% (w/v) BSA and 0.1% (v/v) Triton X-100 to determine the amounts of DPD and OPRT. The protein contents of the tissue extracts were colorimetrically determined at 490 nm using the BCA protein reagent.

Crude enzyme extracts and standard proteins were added to the wells of the TS, DPD and OPRT-ELISA plates, and colorimetric measurement of the optical density (OD) at 490 nm was made using a plate-reader (Biokinetic Reader EL 340; Bio-Tec Inc., USA). The amounts of TS, DPD and OPRT in the tumor extracts were calculated as nanograms of TS, DPD

Table I. Assayed specimens summarized by cancer types.

	mRNA		Protein expression	
	T	N	T	N
Head and neck cancer (HNC)	200	88	399	114
Gastric cancer (GC)	826	27	1824	1127
Colorectal cancer (CRC)	1691	257	3124	1923
Pancreatic cancer (PC)	39	0	62	46
Breast cancer (BC)	373	0	1832	1439
Lung cancer (LC)	816	6	207	190
Others	386	121	287	209
Total	4331	499	7735	5048

Others contain biliary tract cancer, esophageal cancer, prostate cancer and hepatocarcinoma.

or OPRT enzyme per milligram of protein using the standard curves for TS, DPD and OPRT, respectively. The protein expression determined by the ELISAs in this study were correlated with the enzyme activities (21,28).

Statistics. The Spearman's rank correlation analysis was used to evaluate the correlations among the mRNA and protein expression levels of TS, DPD and OPRT. The significances of the differences in the mean TS, DPD or OPRT expression levels between the surgically resected tumor tissues and the non-cancerous tissues obtained from patients with head and neck, gastric, colorectal, breast, lung and pancreatic cancers were statistically assessed using the Wilcoxon test; JMP version 5.1 package software was used for the analyses.

Results

TS mRNA and protein expression levels. The overall median TS mRNA and protein expression levels in the cancers examined were 2.06 TS/ACTB mRNA and 22.1 ng/mg of protein, respectively (Table II). The relative expression of TS in the various cancers examined varied in the range of 1.45-2.47, and the TS mRNA expression level tended to be the highest in gastric cancers among all the cancers examined. The relative amounts of TS protein varied in the range of 10.9-30.7 ng/mg of protein in the various cancers examined, with the highest amount observed in head and neck cancers. The lowest TS mRNA and protein expression levels were seen in the pancreatic cancer specimens. Comparison of the TS protein expression among matched tumor and non-cancerous tissues revealed significantly higher TS expression levels ($P<0.05$) in head and neck, gastric, colorectal, breast, lung and pancreatic cancer tissues than in the corresponding normal tissue specimens obtained from the same patients (Fig. 1).

DPD mRNA and protein expression levels. The overall median DPD mRNA and protein expression levels in the cancers examined were 0.803 DPD/ACTB mRNA and 134.8 ng/mg of protein, respectively (Table II). The relative mRNA and protein expression in the various cancer specimens examined varied in the range of 0.34-1.87 DPD/ACTB and 111.2-264.0 ng/mg of protein, respectively. The highest and lowest DPD mRNA and protein expression levels were observed in lung cancers and colorectal cancers, respectively. Head and neck cancers and pancreatic cancers showed the second highest levels of DPD mRNA and protein expression. The median DPD mRNA and protein expression

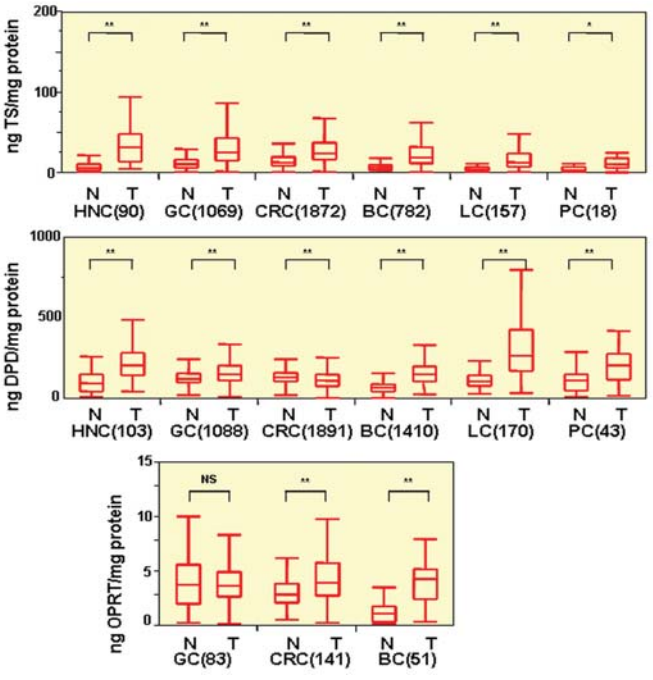

Figure 1. The protein expression of TS, DPD and OPRT in tumor tissues (T) and matched non-cancerous tissues (N) obtained from patients with head and neck, gastric, colorectal, breast, lung and pancreatic cancer. The TS, DPD and OPRT protein expression levels were determined in the tumors and matched non-cancer tissues. The symbols indicate significance, as determined using a paired-analysis Wilcoxon's test. $^{*}p<0.05$; $^{**}p<0.001$.

Table II. mRNA expression and enzyme amount of TS, DPD and OPRT.

	mRNA (relative gene expression)			Protein expression (ng/mg protein)		
	TS	DPD	OPRT	TS	DPD	OPRT
N	4200	3804	4080	7568	7715	480
Mean±SD	2.81±3.13	1.34±1.79	1.52±1.79	34.9±53.8	159.8±116.3	4.30±2.85
Median	2.06	0.803	1.17	22.1	134.8	3.81

mRNA expression based on the internal reference gene ACTB.

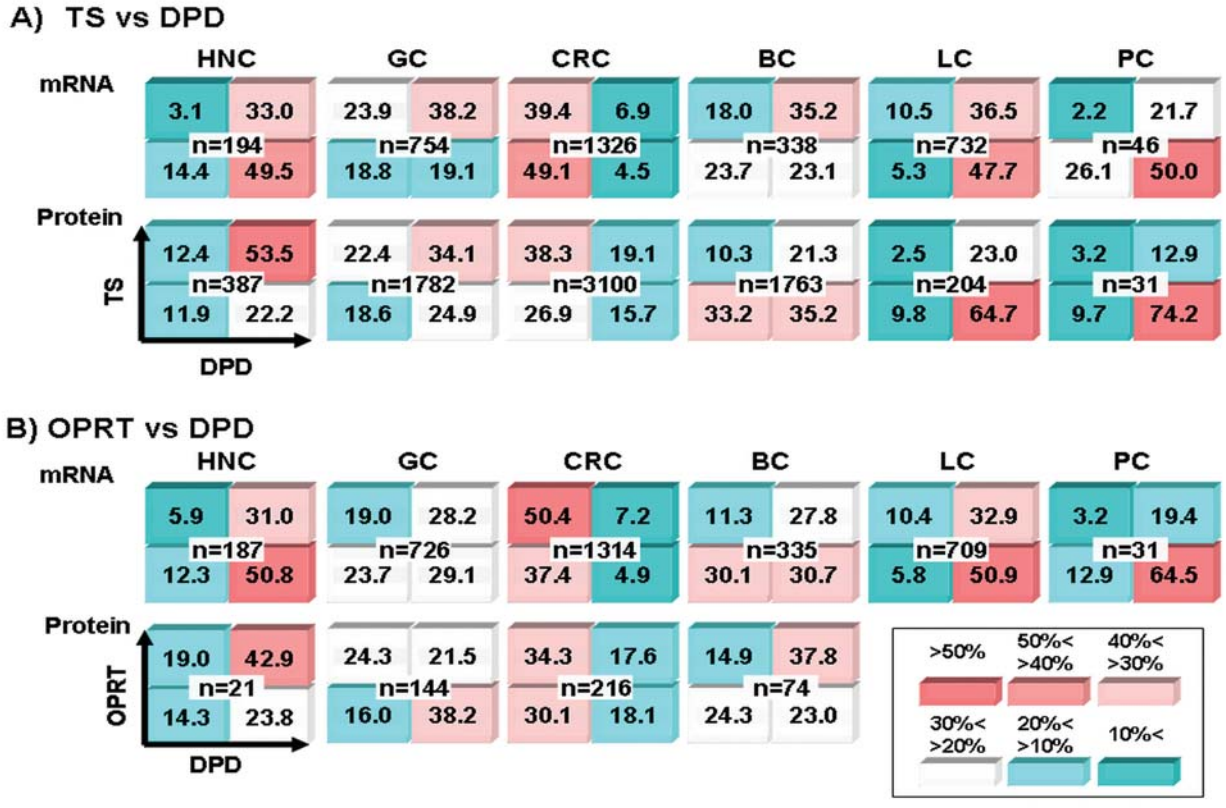

Figure 2. Population analysis based on four categories divided according to the median TS and DPD levels (A) or the median OPRT and DPD levels (B). The percentages of tumors in each category are shown in each of the columns, and the number of specimens analyzed are shown in the center. Populations with high percentages are shown in red, while those with low percentages are in blue (color detail were shown in box). The overall median TS, DPD and OPRT measurements were used as the cutoff values (2.061 TS/ACTB, 0.8030 DPD/ACTB and 1.170 OPRT/ACTB for mRNA expression; 22.10 ng TS/mg of protein, 134.8 ng DPD/mg of protein and 3.806 ng OPRT/mg of protein for protein expression).

levels in the head and neck cancers were 1.36 DPD/ACTB and 207.2 ng/mg of protein, respectively. Among the pancreatic cancers, the median DPD mRNA and protein expression levels were 1.14 and 183.6 ng/mg of protein, respectively. Paired analysis of the DPD protein expression levels between tumor tissues and the corresponding normal tissues revealed significantly higher expression levels of the protein in head and neck, colorectal, breast, lung, and pancreatic cancers ($P<0.001$) than in the corresponding normal tissues.

OPRT mRNA and protein expression levels. The overall median OPRT mRNA and protein expression levels in the cancers examined were 1.17 OPRT/ACTB mRNA and 3.81 ng/mg of protein, respectively (Table II). The relative OPRT mRNA and protein expression varied in the range of 0.80-1.29 OPRT/ACTB and 3.43-4.60 ng/mg of protein, respectively. As shown in Fig. 3, no significant differences in the mRNA and protein expression levels of OPRT were observed among the cancers examined. The expression levels of OPRT protein in the tumor tissues and matched normal tissues are shown in Fig. 1. Significantly higher OPRT protein expression levels were observed in colorectal and breast cancers than in the corresponding normal tissues ($p<0.001$).

Table III. Correlation of TS, DPD and OPRT expression.

	mRNA (relative gene expression)		Protein expression (ng/mg protein)	
	DPD	OPRT	DPD	OPRT
TS	0.0798 (p<0.0001)	0.437 (p<0.0001)	0.0317 (p=0.0058)	0.194 (p<0.0001)
DPD	_	-0.0312 (p=0.0589)	_	-0.0387 (p=0.3971)

Spearman rank correlation coefficient.

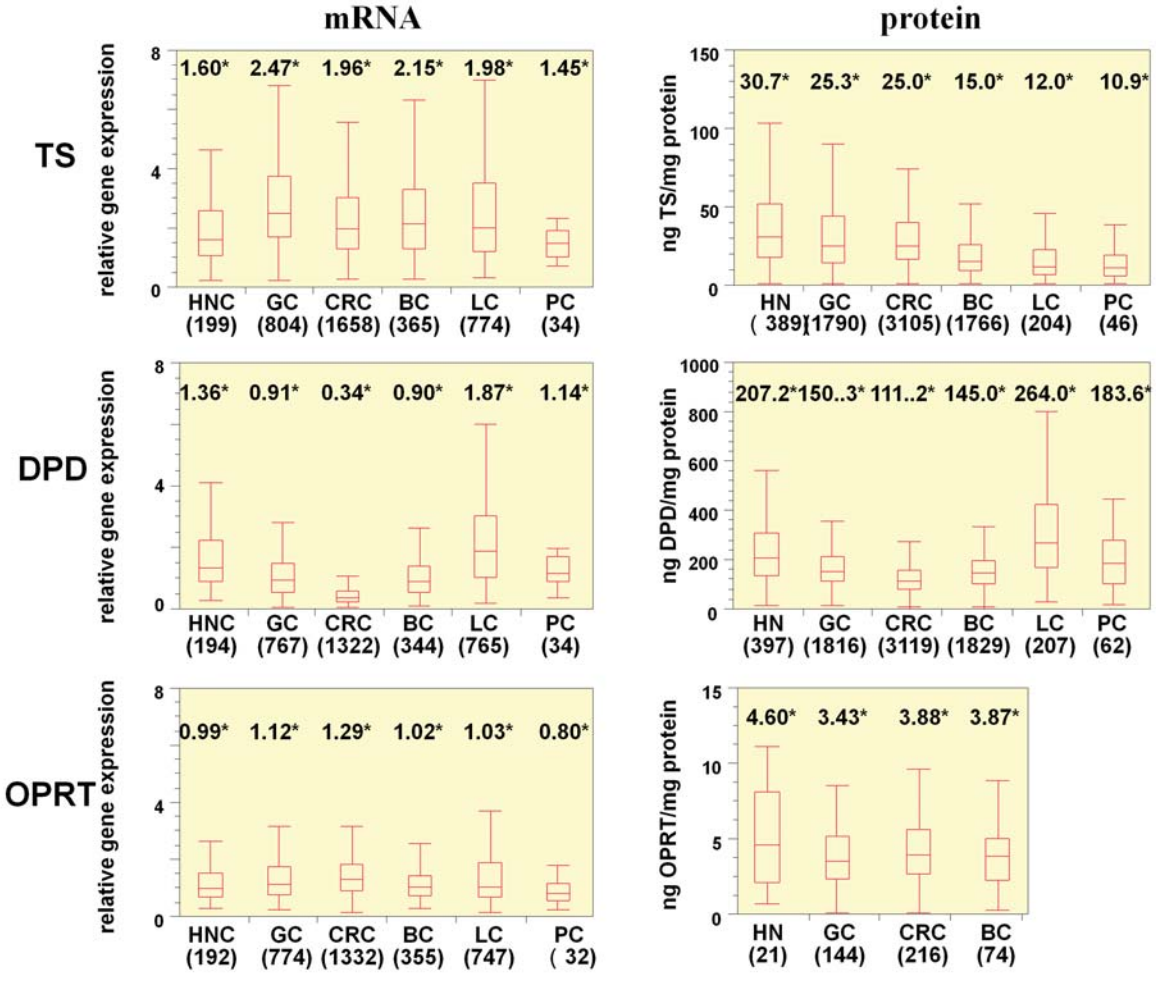

Figure 3. mRNA and protein expressions levels of TS, DPD and OPRT by the cancer types. The number of specimens analyzed is indicated in parentheses. *The median values of TS, DPD and OPRT expression by the cancer types.

Relationship between TS, DPD and OPRT. Table III shows the correlations among TS, DPD and OPRT mRNA and protein expression levels in paired specimens for all the cancers examined. The mRNA expression levels of TS were moderately correlated with those of OPRT (Spearman's rank correlation coefficient: 0.437, p<0.0001), and the TS protein levels were weakly correlated with those of OPRT (Spearman's rank correlation coefficient: 0.194, p<0.0001). No correlations were observed in either the mRNA or protein expression levels between TS and DPD or between DPD and OPRT.

Classification analysis of TS, DPD and OPRT mRNA and protein expression. The median mRNA expression levels of TS, DPD and OPRT in all the tumors measured in this study were 2.06 TS/ACTB, 0.803 DPD/ACTB and 1.17 OPRT/ACTB, respectively, while the median protein expression levels of TS, DPD and OPRT were 22.1, 134.8 and 3.81 ng/mg of protein, respectively. These median values were used to divide the tumors into two sets of four groups each, the first set comprised a low TS/low DPD group, a low TS/high DPD group, a high TS/low DPD group, and a high TS/high DPD group, and the second set comprised a low

OPRT/low DPD group, a low OPRT/high DPD group, a high OPRT/low DPD group and a high OPRT/high DPD group (Fig. 2). More than 80% of the head and neck cancers showed high levels of DPD mRNA expression, and 53.5% were categorized into the high TS/high DPD group with regard to protein expression. Similarly, >57% of the gastric cancers, >84% of the lung cancers, and >71% of the pancreatic cancers exhibited high mRNA and protein expression levels of DPD. In contrast, >65% of the colorectal cancers showed low DPD expression levels, and 49.1% were categorized into the low TS/low DPD group with regard to the mRNA expression. Among the lung cancers, 47.7 and 64.7% were categorized into the low TS/high DPD mRNA and protein expression groups, respectively. Furthermore, 50.0% and 74.2% of the pancreatic cancers were categorized into the low TS/high DPD mRNA and protein expression groups, respectively. In regard to the OPRT and DPD expression, 50.8% of the head and neck cancers were categorized into the high DPD/low OPRT group with regard to the mRNA expression. Concerning the protein expression, 38.2% of the gastric cancers were categorized into the low OPRT/high DPD group; for mRNA expression, 30.7% of the breast cancers, 50.9% of the lung cancers and 64.5% of the pancreatic cancers were categorized into the low OPRT/high DPD group. Furthermore, 50.4 and 34.3% of the colorectal cancers were categorized into the high OPRT/low DPD mRNA expression and protein expression groups, respectively.

Discussion

TS, the rate-limiting enzyme in the *de novo* DNA biosynthetic pathway, has been suggested to play a critical role in 5-FU-based chemotherapy as a sensitivity-predicting factor. Actually, the results of a number of clinical studies have suggested poorer clinical response to 5-FU-based chemotherapy showing high expression levels of TS in the cancer. DPD, a key enzyme for 5-FU degradation, has been referred to as an important enzyme in relation to prediction of the response to 5-FU in clinical studies. In addition, the high 5-FU sensitivity group had higher activity levels of OPRT, a major enzyme in the phosphorylation pathway of 5-FU in human cancers than the low sensitivity group.

Many reports have discussed the relationships among the TS, DPD and OPRT expression levels and the determination of the clinical efficacy of chemotherapy based on the original cutoff values (6-9,15,17,22,23). Due to the lack of reference data on the expression levels of these enzymes in cancer tissues, the cutoff values in various cancers have remained unclear. Moreover, the response to 5-FU based chemotherapy is well known to differ among cancers, and the expression levels of these enzymes also seem to show large inter-individual differences. In this report, we described the differences in the mRNA and protein expression levels of TS, DPD and OPRT by the cancer types, to establish reference data for the Japanese population.

In a previous study, Salonga and colleagues claimed that colorectal cancer patients showing high TS, DPD, and TP expression levels in the tumors showed poor clinical responses and shorter survival times than those showing lower expression levels (32). Similarly, metastatic colorectal cancer patients with high TS and high DPD mRNA expression levels showed shorter survival times following 5-FU chemotherapy (6). Previously, Ichikawa *et al* reported that colorectal cancer patients with a high OPRT/DPD ratio survived longer after 5-FU treatment than those with lower values of the ratio (23). A correlation between the tumor growth inhibition rate following the administration of oral uracil/tegafur (UFT) and OPRT activity has also been reported (21). Therefore, 5-FU based chemotherapy for colorectal cancer seems to be more effective in patients with lower TS, lower DPD and higher OPRT expression levels. In this population analysis, colorectal cancers were found to exhibit lower TS, lower DPD and higher OPRT expression compared with other cancers. The results of this study also indicate, therefore, the expected high efficacy of 5-FU based chemotherapy against colorectal cancer. Numerous regimens for colorectal cancer containing 5-FU, e.g., FOLFOX, the Mayo regimen and FOLFILI, have been established.

In contrast, our classification indicated high DPD expression in most gastric, lung and pancreatic cancers, and these cancers have been reported in clinical practice, to show poor responses to 5-FU based chemotherapy. Easy degradation of 5-FU in the cancer tissues with high DPD expression levels seemed to be one reason of the clinical resistance of these cancers to 5-FU based chemotherapy. To improve the clinical responses to chemotherapy in patients with cancers showing high DPD expression levels, it may be important to control the DPD activity in cancer tissues.

Regarding the regulation of DPD activity in cancer cells, Takechi *et al* demonstrated that the cytotoxicity of 5-FU in high-DPD-expressing cells was considerably potentiated by the addition of 5-chloro-2,4-dihydroxypyridine (CDHP), a potent inhibitor of DPD, *in vitro* and *in vivo* (33). S-1, a DPD-inhibitory fluoropyrimidine (DIF), elicited a 45% response rate in a phase II study in advanced and recurrent gastric cancer (34,35). A phase II study for stage IIIB/IV non-small cell lung cancer reported a response rate of 22% and a median survival time of 10.2 months. The authors concluded that S-1 was active as a singly administered agent against non-small cell lung cancer (36). Moreover, although a low efficacy of 5-FU has been reported in patients with pancreatic cancer (37), a phase II study of S-1 for advanced pancreatic cancer reported a 37.5% response rate (38).

These results suggest that a DIF, such as S-1, might be effective against high-DPD-expressing cancers, including lung, pancreatic and gastric cancers, by inhibiting the tumoral DPD.

In this large-scale population study, we measured the mRNA and protein expression of TS, DPD and OPRT in surgically resected cancer specimens from a large number of subjects. Since 5-FU-resistant cancers exhibited high expression levels of DPD, the tumoral DPD level may be an important factor in predicting the effectiveness of 5-FU-based chemotherapy. The results of this large-scale population study of TS, DPD and OPRT mRNA and protein expression levels are expected to serve as valuable reference data for selecting 5-FU-based chemotherapy, and as a useful clinical reference for TS, DPD and OPRT levels in the population.

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Appendix

Collaborated hospitals.

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CC, cancer center; GH, general hospital; H, hospital; MC, medical center; MH, municipal hospital; MU, medical university; NH, national hospital; PH, prefecture hospital; RCH, red cross hospital; U, university.