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ORIGINAL ARTICLE

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Correlation between clinicopathologic factors and kinetics of metabolic enzymes for 5-fluorouracil given to patients with colon carcinoma by two different dosage regimens

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Abstract *Purpose*: Using tumor tissue specimens from colon carcinoma patients given 5-fluorouracil (5-FU) by two different administration methods, we investigated the degree of correlation between clinicopathologic factors and the kinetics of metabolic enzymes for 5-FU. *Methods*: Group A patients received 500 mg/day of 5-FU as a rapid infusion over 2 h for 3 days preoperatively, and group B patients received 500 mg/day of 5-FU as a continuous infusion for 3 days preoperatively. The activities of orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP), uridine phosphorylase (UP), and dihydropyrimidine dehydrogenase (DPD) were measured by separation on ion-exchange filter paper, by high-performance liquid chromatography (HPLC) with UV detection and by HPLC with radioactive flow monitoring, respectively, and the [^{3}H]-5-fluorodeoxyuridine monophosphate binding site in thymidylate synthetase (TS) was determined as an index of the amount of TS using a radiobinding assay. The TS inhibition rate (TSIR) was calculated from the formula: $1–(TS_{free}/TS_{total})\times100$. Finally, 5-FU incorporation into RNA (FRNA) was measured by capillary gas chromatography-mass spectrometry. *Results*: In group A patients, FRNA showed a positive correlation only with OPRT, and TSIR showed positive correlations with OPRT and TP, but a negative correlation with TS. In group B patients, FRNA showed a negative correlation with DPD, and TSIR showed a negative correlation with TS. No difference in TSIR levels was seen between groups A and B. FRNA was higher in group A than in group B, but the difference was not statistically significant. *Conclusion*: The method of administration may influence 5-FU metabolism in colon carcinoma patients.

Keywords Colon carcinoma · Preoperative adjuvant chemotherapy · Metabolic enzymes for 5-FU · FRNA · TSIR

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Introduction

Even though CPT-11 is now approved for the treatment of colon carcinoma, 5-fluorouracil (5-FU) still has a significant role in the treatment of this cancer. However, 5-FU sometimes has limited efficacy, and we therefore believe that patients should be selected after examining metabolic enzymes and other factors to better predict the drug's efficacy. Although a correlation between thymidine phosphorylase (TP) and the efficacy of the fluorouracil class of anticancer drugs has been reported [22, 29], the enzymes and factors which would predict the effects of these drugs are unclear. Thus, in the present study, we administered 5-FU to colorectal cancer patients by either rapid or continuous infusion and assayed the metabolic enzymes orotate phosphoribosyl transferase (OPRT), TP, uridine phosphorylase (UP), thymidylate synthetase (TS) and dihydropyrimidine dehydrogenase (DPD), and also determined the TS inhibition rate (TSIR) and 5-FU incorporation into RNA (FRNA), which influence the kinetics of the 5-FU metabolic pathway, in order to determine the significance of these enzymes and factors in predicting the outcome of the treatment of colon carcinoma patients with fluorouracil-class drugs.

Materials and methods

The subjects were 44 patients with colorectal cancer resected at the Department of Digestive Tract Surgery, Hachioji Medical Center, Tokyo Medical University. The patients included 28 men and 16 women (mean age 64.8 ± 11.1 years). The histologic stages were: I (one patient), II (19 patients), IIIa/IIIb (14 patients), and IV (10 patients). The patients were divided into groups A and B according to the following dosage regimens: 5-FU 500 mg/day as a rapid infusion over 2 h on each of the 3 days preoperatively (group A); and 5-FU 500 mg/day as a continuous infusion for 3 days preoperatively (group B). Group A included 12 men and 5 women (mean age 68.6 ± 10.1 years). Their histologic stages were: II (6 patients), IIIa/IIIb (5 patients), and IV (6 patients). Group B consisted of 16 men and 11 women (mean age 62.4 ± 11.3 years). Their histologic stages were: I (one patient), II (13 patients), IIIa/ IIIb (9 patients), and IV (4 patients) (Table 1).

OPRT activity was determined using a paper disc method [13]. Briefly, tumor specimens were homogenized in a twofold volume of 50 m*M* Tris-HCl buffer (pH 7.5) containing $MgCl_2$ and dithiothreitol and then centrifuged (10,5000 *g* for 1 h at 4°C), and the supernatant was collected. A solution containing 1.6 μmol [^{3}H]-5-FU (2.5 nCi), 2 μmol 5-phosphoribosyl 1-pyrophosphate, 6 μmol *β*-glycerophosphate, 240 nmol *α*,*β*-methylene adenosine 5′-diphosphate in a total volume of 200 μl was added to 200 μl supernatant maintained at 37°C and incubated. At 5, 10, and 15 min after initiating incubation, 90 μl of the reaction solution was pipetted into a new tube and heated in boiling water to stop the reaction. The reaction mixture, which formed a precipitate on heating, was centrifuged to separate the supernatant. An aliquot (20 μl) of the supernatant was spotted onto a filter paper disc of DEAE-cellulose, and the disc was repeatedly washed to remove nonreacted [^{3}H]-5-FU. To the disc dried in a scintillation vial was added 8 ml Scintisole EX-H (Wako, Tokyo, Japan), and the radioactivity was measured. The OPRT activity was calculated from the amount of 5-fluorouridine monophosphate (FUMP) produced which was proportional to the radioactivity, and the protein concentration in the enzyme solution was measured by the method of Lowry et al. [16].

The activities of TP and UP were determined as follows. Tumor specimens were homogenized in a fourfold volume of 50 m*M* phosphate buffer (pH 7.4) containing 2-mercaptoethanol (2-ME) and then centrifuged (10,5000 *g* for 1 h at 4°C), and the supernatant was collected. The precipitate resulting from the gradual addition of saturated $(NH_4)_2SO_4$ solution to the supernatant was separated by centrifuging and decanting. The precipitate was dissolved in 20 m*M* Tris-HCl buffer (pH 7.4) containing 2-ME (buffer A) and dialyzed overnight against buffer A. After dialysis, the protein concentration of the inner solution in the dialyzer was determined by the method of Lowry et al. [16]. Dialysis solutions with a protein concentration higher than 0.5 mg/ml were diluted with buffer A to 0.5 mg/ml and those with a concentration less than 0.5 mg/ml not diluted.

For determination of TP activity, 50 μl of diluted dialysis solution was added to 750 nmol 5-FU and 750 nmol ribose 1-phosphate in a total volume of 100 μl. After 5 min incubation at 37°C, the reaction was terminated by adding 35 μl 3 *M* $HClO_4$, and 25 μl 220 μ*M* 3-methylxanthine as internal standard was added to the mixture. After removal of the precipitate by centrifugation, the supernatant was used as the sample solution for high-performance liquid chromatographic (HPLC) analysis. Sample solutions for determination of UP activity were prepared in the same manner as for the TP activity assay except for the use of 2′-deoxyribose 1-phosphate instead of ribose 1-phosphate and 15 min incubation. The solutions were injected into a YMC-Pack ODS-AM HPLC reversed-phase column (AM-302; YMC, Kyoto, Japan). Elution was performed using a mobile phase consisting of water, methanol, and acetonitrile (970:15:15) at a flow rate of 0.8 ml/min. The eluate was monitored at an absorbance of 269 nm, and the amount of fluorodeoxyuridine and 5-FU produced was measured to determine the activities of TP and UP, respectively.

TS forms a stable ternary complex in vivo and in vitro together with 5-fluorodeoxyuridine monophosphate (FdUMP) and methylene tetrahydrofolic acid. Spears et al. quantitated [^{3}H]-FdUMP binding sites present in TS using this property [24]. In our study, the TS assay was performed by the method of Spears et al. with some modifications. In brief, tumor tissue was homogenized in a fourfold volume of 50 m*M* phosphate buffer (pH7.4) containing NaF, cytidine 5′-monophosphate and 2-ME, and centrifuged. The supernatant was then divided into two portions as the cytosol for the determination of TS_{total} and TS_{free} (TS_{total} is the sum of [^{3}H]-FdUMP binding sites in TS not yet forming complex and TS made to dissociate to FdUMP from the complex formed in vivo by administration of 5-FU).

TS_{total} was determined as follows. To 50 μl 50 m*M* Tris containing NaF, cytidine 5′-monophosphate and 2-ME (pH 8.0) was added 50 μl of the cytosol, and the resulting solution incubated for 3 h at 25°C to dissociate FdUMP from the complex. After incubation, 9.5 pmol [^{3}H]-FdUMP (0.15 mCi) and 25 μl of a solution containing tetrahydrofolic acid, sodium ascorbate and formaldehyde were added, followed by incubation for 20 min at 25°C. To this solution was added 1 ml of a cold slurry comprising 3.3 g activated charcoal in 100 ml 0.1 *M* HCl containing dextran and bovine serum albumin, and the suspension was allowed to stand for at least 40 min in an ice-water bath. The [^{3}H]-FdUMP-bound ternary complex was separated by centrifugation as the supernatant, 850 μl of which was transferred to a scintillation vial and mixed with 8 ml Scintisole EX-H, and the radioactivity determined with a liquid scintillation counter. The same procedure was used for purified *Lactobacillus casei* TS (Biopure, Boston, Mass.) with previously quantitated [^{3}H]-FdUMP binding sites as the standard protein. Thus, the sum of [^{3}H]-FdUMP binding sites in tissue samples, TS_{total}, was calculated from the standard curve based on *L. casei* TS. TS_{free} was determined in the same manner as TS_{total}, except without incubation for 3 h at 25°C. Further, the inhibition rate of TS activity (TSIR) with FdUMP was calculated using the formula $[1–(TS_{free}/TS_{total})]\times100$.

DPD activity was assayed using HPLC with a radioactive flow monitor [8]. Briefly, tumor tissue was homogenized in 20 m*M* phosphate buffer (pH 8.0) containing EDTA and 2-ME, and then centrifuged (105,000 *g* for 1 h at 4°C), and the supernatant was collected and its protein concentration determined by the method of Bradford [3]. To 250 μl of the supernatant was added 12.5 μl 6.25 m*M* NADPH solution and 50 μl 125 μ*M* [^{3}H]-5-FU (1.25 μCi), and the mixture incubated at 37°C for the enzyme reaction. At 10, 20, and 30 min after initiating incubation, an aliquot (70 μl) of the reaction mixture was pipetted and mixed with an equal volume of 5% $HClO_4$. The mixture was then mixed with 140 μl of the mobile phase for HPLC analysis and centrifuged. The supernatant was used as the sample solution for HPLC analysis. The sample solution was injected onto the HPLC reversed-phase column (YMC-Pack Pro C18, AS-301-3, YMC). The elution was performed using 20 m*M* sodium phosphate buffer (pH 3.5) as mobile phase at a flow rate of 0.5 ml/min. 5-FU and its metabolites in the reaction mixture were monitored by a radioactive flow monitor flowing the eluate together with Pico-Fluor 40 (Packard, Meridien, Ct.). DPD activity was calculated as the enzyme reaction rate per unit amount of protein (1 mg) in the reaction solution.

Table 1 Patient details

	Group A	Group B
No. of patients		
Men	12	16
Women	5	11
Age (years, mean ± SD)	68.6 ± 10.1	62.4 ± 11.3
Stage (no. of patients)		
I	0	1
II	6	13
IIIa/IIIb	5	9
IV	6	4

FRNA was assayed using gas chromatography-mass spectrometry (GC-MS) [18] with some modifications. In brief, 50 to 100 mg of tissue specimen was homogenized in 2 ml water, mixed with 5 ml cold 5% trichloroacetic acid (TCA), and centrifuged. The resulting precipitate was washed twice by mixing with each of 5 ml cold 5% TCA, 70% ethanol, 95% ethanol, and ethanol/diethyl ether (3:1) solvent, followed by centrifugation and discarding the supernatant. The final precipitate was dissolved in 0.3 *M* KOH, and incubated overnight at 37°C to hydrolyze RNA to mononucleotide. After neutralizing with $HClO_4$ and desalting, a portion of the mononucleotide solution was used for the determination of the concentration of RNA in terms of the color reaction between mononucleotide and orcinol and standard RNA from bakers yeast. To 1.4 ml of the residual mononucleotide solution was added 100 µl of 1 µg/ml [$^{15}N_2$]-5-FU as internal standard, and this solution was mixed with an equivalent volume of 12 *M* HCl and hydrolyzed in a closed tube for 20 h at 100°C. After cooling and washing the reaction solution with $CHCl_3$, the solution was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 *M* phosphate buffer (pH 4.0) and extracted with ethyl acetate. The extract was purified by preparative silica gel column chromatography. The residue of the fraction containing 5-FU and internal standard was dissolved in 30 µl acetonitrile, and reacted with 10 µl ditrifluorobenzyl bromide in the presence of triethylamine for 20 min at room temperature. Then 50 µl ethyl acetate followed by 450 µl *n*-hexane was added to the reaction mixture, and a precipitate formed in the solution. After centrifugation of the solution, the supernatant was transferred to a new tube. The solvent was removed under a stream of nitrogen, and the residue was reconstituted with 200 µl *n*-hexane as the sample solution for GC-MS.

GC-MS analysis was carried out with a system consisting of a Hewlett Packard 5890 gas chromatograph and a JEOL Automass JMS-AM150 mass spectrometer. To the gas chromatograph was connected a DB-1 fused silica capillary column, 30 m×0.25 mm internal diameter (J & W Scientific, Folsom, Calif.). The GC conditions were as follows: the carrier gas was helium at a flow rate of 1.0 ml/min at the outlet of column; the injector temperature was 250°C; the interface temperature 250°C; and the oven temperature was maintained at 100°C for 1 min, then programmed to increase at 20°C/min to 300°C, and maintained at 300°C for 10 min. An aliquot (1 µl) of the sample solution was injected into the GC-MS in splitless mode. The mass spectrometer was operated under negative ion chemical ionization mode. Isobutane was introduced into the ion source at about 0.5 Torr as reagent gas. The source temperature was 150°C, and the ionization energy 150 eV. 5-FU and internal standard were monitored with ions of m/z 355 and 357, respectively. The analysis was based on an established procedure. The apparatus for analysis was regularly checked with QC samples. We calculated the mean ± SD values from the analytical data for each item to be used as the supplemental data for validation.

We explained to patients that preoperative administration of 5-FU was to predict the response to postoperative 5-FU and obtained their informed consent. This study was approved by the committee on ethics and clinical studies of our institution.

For statistical analysis, the *t*-test was used to compare the activities of the metabolic enzymes between groups. Correlations between metabolic enzymes and TSIR and between the enzymes and FRNA were evaluated by Spearman's test, and correlations between the enzymes and clinicopathologic factors were evaluated by the Tukey-Kramer test. Differences were considered statistically significant with *P* values less than 0.05.

Results

FRNA and OPRT showed a positive correlation ($P < 0.05$) in group A. TSIR showed positive correlations with OPRT and TP in group A, and TSIR and TS showed a negative correlation ($P < 0.05$) in groups A and B (Table 2). FRNA and DPD showed a negative correlation ($P < 0.05$) in group B. Although TSIR and TS_{free} showed a negative correlation ($P < 0.01$) in groups A and B, no correlation was observed between TSIR and the other enzymes investigated (Table 2). No differences were seen in the activities of the metabolic enzymes between groups A and B and there were no differences in TSIR between the groups. FRNA was higher in group A than in group B, but the difference was not significant.

The results of our investigation of correlations between enzyme activities and clinicopathologic factors in 44 patients showed significant differences ($P < 0.05$) in the enzyme activities in relation to stage: UP 6.15 ± 2.7 nmol/min per mg protein in Dukes B, and 11.5 ± 6.3 nmol/min per mg protein in Dukes C. However, there were no correlations between the clinicopathologic factors histologic type, degree of invasion of the colorectal wall, lymphatic/vascular permeation, lymph node metastasis and distant metastasis and the activities of OPRT, UP, TP, TS and DPD (Table 3).

Discussion

Although a metabolic pathway in which, in order to show an antitumor effect, 5-FU is phosphorylated by FU nucleotide has been reported [30], the details of the main kinetic pathway of the drug are unclear [17] resulting in insufficient use of the finding, especially clinically. This is partially due to the fact that animal

Table 2 5FU metabolic enzyme activities and their correlation with FRNA and TSIR

	Group A	Group B	Correlation with FRNA		Correlation with TSIR	
			Group A	Group B	Group A	Group B
OPRT (nmol/min/mg)	0.23 ± 0.19	0.23 ± 0.15	0.632*	–0.045	0.573*	0.239
TP (nmol/min/mg)	11.87 ± 6.77	14.65 ± 6.19	–0.021	0.103	0.573*	0.064
UP (nmol/min/mg)	5.18 ± 2.30	6.21 ± 2.40	–0.006	–0.053	0.405	–0.033
TS_{free} (pmol/g)	14.20 ± 18.23	10.88 ± 7.36	–0.321	0.394	–0.573**	–0.676**
DPD (pmol/min/mg)	28.00 ± 19.50	26.0 ± 14.7	0.109	–0.609*	0.084	0.226
TSIR (%)	58.57 ± 14.2	53.56 ± 15.81	–	–	–	–
FRNA (ng/mgRNA)	117.2 ± 65.3	78.6 ± 44.0	–	–	–	–

*$P < 0.05$, **$P < 0.01$; Spearman's test

Table 3 Correlations between metabolic enzymes and clinicopathologic factors. As indicated, the only significant correlation was seen for UP between stage II and stage IIIa/IIIb; no other significant correlation was observed

	OPRT	TP	UP	TS free	DPD
Histologic differentiation					
High	0.25	14.36	6.28	12.57	26.67
Moderate	0.21	16.22	8.34	10.06	23.35
Poor	0.20	25.40	9.35	3.70	33.00
Others	0.17	5.40	2.99	4.25	25.00
Invasion to the wall					
Submucosal layer	0.23	3.12	1.67	7.00	8.00
Proper muscle layer	0.16	14.90	8.69	11.05	26.75
Subserous layer	0.27	12.33	7.55	11.45	24.91
Serous coat	0.22	17.16	7.45	–	26.05
Vascular permeation					
0	0.26	19.57	7.71	11.60	30.25
1	0.22	14.75	7.51	11.95	20.00
2	0.23	15.53	7.52	7.24	30.50
3+	0.27	11.48	5.39	9.60	27.67
Lymph node metastasis					
0	0.23	13.40	5.20	12.35	19.27
1	0.19	17.41	7.32	10.22	31.70
2	0.36	13.75	10.71	7.63	26.22
3+	0.12	25.40	9.35	3.70	34.00
Stage					
I	0.23	3.12	1.67	12.97	8.00
II	0.25	14.51	6.15	9.00	20.07
IIIa/IIIb	0.22	20.69	11.51*	6.74	31.69
IV	0.18	12.50	5.28	–	26.13

*$P < 0.05$, vs stage II; Tukey-Kramer's test

experiments have shown two different cytocidal modes of action of 5-FU resulting from different administration methods: higher 5-FU concentrations with shorter duration of action, and lower 5-FU concentrations with longer duration of action [7]. Another reason why the metabolic kinetic pathway of 5-FU remains uncertain is due to the difficulty of assaying the metabolic enzymes of the drug clinically. 5-FU is metabolized by OPRT, TP and UP to generate FRNA which is thought to damage RNA synthesis, though impairment of RNA synthesis also results from such factors as uridine kinase as well as cosubstrates, i.e. phosphoribosyl pyrophosphate and ribose-1-phosphate [6]. Furthermore, DNA damage may be due to inhibition of TS through the metabolic pathway of TP or from UP to 5-fluorouridine diphosphate (FUDP) and FdUMP.

However, impairment of DNA synthesis is thought to result from the fact that RNA synthesis, which generates FRNA, is damaged [1]. It has also been suggested that the 5-FU metabolic pathway includes an inactivating pathway involving incorporation of the drug by DPD, indicating that DPD plays an important role [5].

Regarding the dose and administration period of 5-FU, we used a dose of 500 mg/patient, which has been reported by Lokich et al. [15] to be effective, for 3 days in order to avoid side effects and variations in tissue concentrations, bearing in mind the short elimination half-life of 5-FU.

Most studies have dealt with the correlation between TP and 5′-DFUR, and have indicated that tumors with higher levels of TP have higher sensitivity to anticancer drugs [25, 27]. Previous studies have shown that, in colorectal cancers, TP is characteristically present at higher levels in the stroma than in the cancer cells [26], the degree of staining of cancer cells correlates with cancer grade [11], and the degree of staining of stroma correlates with angiogenesis [21]. Recent studies have shown a reciprocal relationship between TP expression and clinicopathologic factors [19] suggesting disagreement among the findings. Other studies have shown that high DPD activity leads to decomposition of fluoropyrimidine drugs [5, 20], and higher levels of TS tend to lead to a decrease in TSIR resulting in lower effects of 5-FU [2, 10, 23].

Since these studies, based on a single factor, are limited in their ability to predict drug efficacy, we tried to evaluate drug efficacy by assessing all enzymes in relation to the method of administration. As in a similar study by Uchida and Nakamura [28], we also considered OPRT. This is because the metabolic pathway to generate active nucleotide in association with UP and TP is not the predominant pathway due to lower activities of uridine kinase and thymidine kinase. Thus, we sought to determine the pathway associated with the direct generation of a nucleotide, i.e. FUMP, from OPRT, as this is a more significant pathway for 5-FU efficacy. Furthermore, since FdUMP is generated more slowly in the pathway in which TP is present, another metabolic pathway from FUDP to ribonucleotide reductase may be important [9].

The present study disclosed that rapid infusion of 5-FU resulted in a significant positive correlation between FRNA and OPRT. The pathway associated with OPRT is considered an important route because of the abundant expression of phosphorylating enzyme notwithstanding lower OPRT expression. This was also proven in the present study. Since the present study also showed a positive correlation between TSIR and OPRT as well as TP, a pathway associated with OPRT from FUDP to fluorodeoxyuridine diphosphate (FdUDP) and FdUMP was confirmed as an important route, which supports the findings of Fukushima et al. [6].

The present study of continuous infusion 5-FU showed a significant correlation between FRNA and DPD, suggesting that the efficacy of 5-FU is mainly due to anti-DNA activity together with limited anti-RNA activity, that 5-FU is predominantly metabolized through the pathway from FUDP to FdUMP, and that DPD, an inactivating enzyme against 5-FU, is more readily influenced by continuous infusion than by rapid infusion of 5-FU. In the 5-FU metabolic pathway, a characteristically slow generation of the FU nucleotide was found, suggesting possible nucleotide generation also by other metabolic pathways. This may be a reason why a definite pathway for 5-FU was not sufficiently proven in the present study.

Furthermore, continuous 5-FU infusion was strongly related to TS in every aspect of drug metabolism, and,

similar to a previous report [14], the activity of TS and the efficacy of fluorinated pyrimidine drugs were also proven. These findings suggest that the antitumor efficacy of 5-FU is mainly based on anti-DNA activity by the covalent ternary complex, that 5-FU is predominantly metabolized by a metabolic pathway to generate FRNA, and that FdUMP is mainly generated by the metabolic pathway from FUDP to FdUDP. The results of the present study also showed that, regarding FRNA generation and TSIR, 5-FU by rapid infusion is predominantly metabolized by the OPRT pathway and is also associated with TP.

When 5-FU was given by drip infusion, the route for generation of FRNA, especially of OPRT, strongly relates to the kinetics of FRNA and TSIR, and when 5-FU was administered continuously, 5-FU metabolism was predominantly influenced by DPD, and following either administration method TSIR was strongly related to TS. These findings led us to conclude that, to prevent decomposition, 5-FU should be injected by rapid short-duration infusion in order to generate FRNA to cause RNA damage, and to cause DNA damage by FdUMP generated from FUDP and FdUR. This results in the most efficient 5-FU metabolism, and concomitant administration of a biochemical modulator, which accelerates the formation of the covalent ternary complex, is thought to be the most efficient dosage regimen [4, 12]. This approach is also possibly valid clinically.

The present study showed no correlation between the metabolizing enzymes and clinicopathologic factors. We have previously reported TP activity in cells and stroma of carcinoma [11]. Further immunohistochemical investigation may clarify correlations between metabolizing enzymes and clinicopathologic factors, recurrence or prognosis of colorectal carcinoma.

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