Gene expression in colorectal cancer and *in vitro* chemosensitivity to 5-fluorouracil: A study of 88 surgical specimens

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To predict the sensitivity of colorectal cancer to 5-fluorouracil (5-FU), we compared the gene expression of surgically obtained colorectal cancer specimens with chemosensitivity to 5-FU as detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay. Eighty-eight patients with advanced and/or metastatic colorectal cancer provided written informed consent and entered the trial from September 2000 to October 2001. Fresh surgical specimens were used for the MTT assay, and sensitivity to 5-FU was evaluated at a cutoff concentration of 50 μg/ml and 48-h incubation time. Frozen samples were stored at −80°C until mRNA analysis of thymidylate synthetase (TS), dihydropyrimidine dehydrogenase (DPD), thymidine phosphorylase (TP), es-nucleoside transporter (NT), and E2F1 by real-time RT-PCR. The correlations between the variables were analyzed, and the predictive value of these mRNAs was assessed statistically using a receiver operating characteristic (ROC) curve. NT and DPD, TP and DPD, and TP and NT mRNA expression levels correlated significantly, while TS and E2F1 showed no correlations. High NT expression was associated with low sensitivity to 5-FU ($P<0.013$), as were high DPD and E2F1 expression ($P<0.022$ for both). High TP mRNA expression correlated with low sensitivity to 5-FU ($P<0.034$), although high TS mRNA expression did not. ROC curves indicated that DPD and NT mRNAs were possible predictors of sensitivity to 5-FU, with cutoff values of 0.6 and 0.4, respectively. The sensitivity of colorectal cancer to 5-FU may be regulated by DPD, the rate-limiting enzyme of catabolism, and NT, an important transmembrane transporter of nucleosides. (Cancer Sci 2003; 94: 633–638)

Colorectal cancer is a major cause of cancer-related mortality. In attempts to improve the outcome of this disease, clinical trials of adjuvant systemic therapy have been performed. The combination of 5-fluorouracil (5-FU) and leucovorin (LV) has been the "standard" therapy for patients with colorectal cancer for over a decade. National Surgical Adjuvant Breast and Bowel Project trials that compared different adjuvant chemotherapy regimens with no adjuvant treatment have reported the relative efficacy of adjuvant chemotherapy in terms of Dukes' staging. Overall, disease-free, and recurrence-free survivals were improved for Dukes' C patients in all four trials.[1–3] Recently, great efforts have been made to improve the efficacy of 5-FU. The third-generation oral 5-FU prodrugs, including orzel, S-1, and capecitabine, may eventually replace infusional 5-FU therapy and improve patients' quality of life by allowing outpatient therapy. Novel combinations of 5-FU or its analogs with agents that have different mechanisms of action (e.g., oxaliplatin, irinotecan) could provide important new opportunities for improving the outlook for patients with colorectal cancer.[4]

When a meta-analysis was conducted on nine randomized clinical trials that compared 5-FU with 5-FU plus intravenous LV in patients with advanced colorectal cancer, 5-FU plus LV showed a highly significant benefit over 5-FU alone in terms of tumor response rate, but this increase in response did not result in a discernable improvement in overall survival. However, a large number of patients did not respond to treatment in both groups.[5] We reported previously that the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay may be useful in evaluating appropriate adjuvant cancer chemotherapy for gastrointestinal cancer, given that drug-sensitive patients showed favorable survival compared to drug-insensitive patients with gastric[6–8] and colorectal[9] cancer. In addition, we reported that the expression of dihydropyrimidine dehydrogenase (DPD) mRNA predicts the sensitivity of human tumor xenografts[10] and human gastric cancer specimens[11] to 5-FU. In the present study, we attempted to assess the sensitivity of 88 colorectal cancer specimens to 5-FU by MTT assay, comparing the mRNA expression levels of thymidylate synthetase (TS), DPD, thymidine phosphorylase (TP), es-nucleoside transporter (NT), and E2F1 by real-time RT-PCR in order to evaluate the usefulness of these mRNAs as predictors of 5-FU sensitivity.

Patients and Methods

Drugs. 5-Fluorouracil (5-FU) was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. All other chemicals used were of the highest standard grade commercially available.

Evaluation of antitumor activity. We evaluated the *in vitro* chemosensitivity of fresh surgical specimens of colon cancer using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) assay as reported by Mosmann,[12] with some modifications.[6–9]

Tissue specimens were obtained during surgery from patients who had given written informed consent. Resected specimens were stored in Hank's balanced salt solution (GIBCO, Gaithersburg, MD) containing 100 IU of penicillin (GIBCO), 100 μg of streptomycin (GIBCO), and 0.25 μg of amphotericin B (GIBCO) per ml, and brought directly to our laboratory. Single-cell suspensions were prepared enzymatically for 30 min using 0.5 mg/ml pronase (Boehringer Mannheim GmbH, Mannheim, Germany), 0.2 mg/ml collagenase type I (Sigma), and 0.2 mg/ml DNase (Sigma). After two centrifugations, tumor cells were suspended in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and then diluted to 1×10^5 cells/ml. Aliquots (100 μl) plated into 96-well microplates (GIBCO) resulted in approximately 10^4 cells per well. Drug solutions were dissolved in RPMI-1640, and 100 μl aliquots were added to each well, giving final concentrations of 50 μg of 5-FU per ml, as previously reported.

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Control wells contained 100 μg of cell suspension and 100 μl of RPMI-1640 containing 10% FBS, while 200 μl of RPMI with 10% FBS was used as a blank. Plates were incubated for 48 h at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. A mixture of 0.4% MTT (Sigma) and 0.1 *M* sodium succinate (Wako Pure Chemical Ind., Ltd., Osaka), each dissolved in 10 μl of phosphate-buffered saline and filtered through a 0.45-mm membrane filter (Millipore, Bedford, MA), was then added, and the plates were incubated for an additional 3 h at 37°C. After the final incubation, 150 μl of dimethyl sulfoxide (Nacalai Tesque, Kyoto) was added to each well to dissolve the MTT-formazan salt, and the plates were shaken mechanically for 10 min on a mixer (Model 250, Sonifier, Branson, MO). Optical densities of each well were determined on a model EAR 340 easy reader (SLT-Labinstruments, Salzburg, Austria) at 540 nm and 630 nm. Inhibition rates (IR, %) were calculated using the following formula: $(1-A/B)\times 100$ (%), where A and B represent the mean absorbance of the treated and control wells, respectively.

Patients. A total of 88 patients with advanced colorectal cancer gave their written informed consent and entered the study. They underwent surgery as well as MTT assay at the Keio University Hospital between September 2000 and October 2001. Surgical specimens were stored at −80°C for gene expression analysis, which was approved by the Ethical Committee of our Hospital (#12–13). Microscopic staging of the tumors was performed according to "The General Rules for the Clinical and Pathological Study of Cancer of the Colon, Rectum and Anus."[13]

RNA extraction and cDNA synthesis. Total RNA was isolated using an RNeasy mini kit (QIAGEN, Inc., Chatsworth, CA), and DNase treatment was performed using the RNase-Free DNase Set (QIAGEN, Inc.), following the manufacturer's instructions.

Reverse transcription with up to 10 μg of total RNA was carried out in a total volume of 100 μl containing 250 pmol of oligo$(dT)_{18}$, 80 U of RNasin ribonuclease inhibitor (Promega, Madison, WI), and 500 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, and 0.5 m*M* dNTPs. Initially, the total RNA solution mixed with oligo$(dT)_{18}$ was heated at 70°C for 10 min and immediately chilled on ice, then the other reagents were added. First-strand cDNAs were obtained after 15 min at 30°C and 60 min at 42°C.

Primers and "*Taq*Man" probes. Primers and "*Taq*Man" probes for TS, DPD, TP, and E2F1 were designed using the Primer Express software (PE Biosystems, Foster City, CA). Primers and *Taq*Man probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from PE Biosystems. The probes were labeled with a reporter dye (FAM) situated at the 5′ end of the oligonucleotide and a quencher dye (TAMRA) located at the 3′ end. The sequences of primers and probes are summarized in Table 1.

PCR procedure. Quantification of target cDNA (TS, DPD, TP, and E2F1) and an internal reference gene (*GAPDH*) was conducted using a fluorescence-based real-time PCR method (*Taq*Man PCR using ABI PRISM 7700 Detection System, PE Biosystems).[14] The PCR was carried out in a final volume of 25 μl containing cDNA equivalent to 1–10 ng of total RNA, 200 n*M* each primer, 100 n*M* probe, and 12.5 μl of *Taq*Man universal PCR Master Mix (containing 1× *Taq*Man buffer, 200 μ*M* dATP, dCTP, dGTP, and 400 μ*M* dUTP, 5 m*M* $MgCl_2$, 1.25 unit of Ampli*Taq*Gold, and 0.5 unit of AmpErase UNG) purchased from PE Biosystems. Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min.

Relative quantification of gene expression. Quantification was performed using the relative standard curve method. The standard curve was created automatically by the ABI PRISM 7700 Detection System by plotting the threshold cycle (C_T) against each input amount (containing 16, 4, 1, 0.25, 0.063, 0.016, 0.0039 ng) of control total RNA (total starting RNA), prepared from MDA-MB-231 human breast tumor cells (American Type Culture Collection, Manassas, VA). The coefficient of linear regression (r) for each standard curve was more than 0.990. For each unknown sample, the relative amount was calculated using linear regression analysis from the respective standard curve. A relative target gene expression value was obtained by division of the target gene value by the value for *GAPDH* as an internal reference gene.

Statistical analysis. Patient clinicopathological factors examined included age, gender, microscopic depth of invasion (T), microscopic lymph node metastasis (N), hepatic and peritoneal me-

Table 1. Sequences of PCR primers and sequence-specific probes for target genes and GAPDH

Gene (GenBank Accession No.)	Primer/probe	Sequence	Corresponding cDNA sequence
TS (X02308)	Forward primer	GAATCACATCGAGCCACTGAAA	882–1099
	Reverse primer	CAGCCCAACCCCTAAAGACTGA	
	Probe	TTCAGCTTCAGCGAGAACCCAGA	
DPD (U09178)	Forward primer	AATGATTCGAAGAGCTTTTGAAGC	1755–1862
	Reverse primer	GTTCCCCGGATGATTCTGG	
	Probe	TGCCCTCACCAAAACTTTCTCTCTTGATAAGGA	
TP (M63193)	Forward primer	CCTGCGGACGGAATCCT	700–770
	Reverse primer	GCTGTGATGAGTGGCAGGCT	
	Probe	CAGCCAGAGATGTGACAGCCACCGT	
E2F1 (M96577)	Forward primer	AGGAGTTCATCAGCCTTTCCC	1325–1426
	Reverse primer	CCCCAAAGTCACAGTCGAAGAG	
	Probe	CCCACGAGGCCCTCGACTACCAC	
NT (AF079117)	Forward primer	TCTTCATGGCTGCCTTTGC	1295–1373
	Reverse primer	GGCTTCACTTTCTTGGGCC	
	Probe	TCGCCAGCCTCTGCATGTGCTT	
GAPDH (M33197)	Forward primer	GAAGGTGAAGGTCGGAGTC	66–291
	Reverse primer	GAAGATGGTGATGGGATTTC	
	Probe	CAAGCTTCCCGTTCTCAGCC	

The probes were labeled with a reporter dye (FAM) situated at the 5′ end of the oligonucleotide and a quencher dye (TAMRA) located at the 3′ end. TS, thymidylate synthetase; DPD, dihydropyrimidine dehydrogenase; TP, thymidine phosphorylase; NT, es-nucleoside transporter.

tastases (M), and histological differentiation. These background factors were compared using the χ^2 test. The coefficients of correlation were evaluated for expression of the various mRNAs, and the significance of differences was confirmed by means of the *t* test.

The sensitivity to 5-FU detected by MTT assay was represented by the IR (%), and compared with mRNA expression, expressed as a ratio to the internal standard, GAPDH. Sensitivity and expression were compared by using coefficients of correlation, and the significance of differences was confirmed by means of the *t* test. When a statistically significant correlation was confirmed, the receiver operating characteristic (ROC) curve was applied to confirm further the predictive value of each mRNA and to obtain the cutoff value for predicting the sensitivity to 5-FU detected by MTT assay.

Results

Eighty-eight specimens were evaluable for MTT assay as well as for measurement of mRNAs. The efficacy rate of 5-FU was 11.3% (10/88), which is similar to the previously reported rate, 11%.[5] The correlation of chemosensitivity to 5-FU and clinicopathological background is shown in Table 2. There was no statistically significant correlation between the 5-FU sensitivity and conventional clinicopathological background factors, except that tumors with distant metastases tended to be more sensitive to 5-FU compared with tumors without distant metastases. The coefficients of correlation for each mRNA are shown in Table 3, where statistically significant correlations were observed between NT/DPD, TP/DPD, and TP/NT mRNAs. In particular, DPD mRNA and TP mRNA showed a very close correlation, with a coefficient of correlation of 0.76 (Fig. 1). TS and E2F1 mRNAs did not correlate with the expression of the other mRNAs.

The correlation between the 5-FU sensitivity of the resected specimens and mRNA expression is shown in Table 4. DPD, NT, TP, and E2F1 mRNAs showed statistically significant correlations with 5-FU sensitivity, represented as IR, at $P<0.05$, while no significant correlations were observed between TS

Table 2. Clinicopathological background and chemosensitivity to 5-fluorouracil

	Sensitivity≥50%	<50%	Total	χ^2
Age >60	6	45	51	n.s.
≤60	4	33	37	
Gender male	7	48	55	n.s.
female	3	30	33	
T 2	0	12	12	n.s.
3	10	61	71	
4	0	5	5	
N 0	6	34	40	n.s.
1	3	26	29	
2	1	18	19	
M 0	8	75	83	0.03
1	2	3	5	
H 0	8	70	78	n.s.
1	0	3	3	
2	2	2	4	
3	0	3	3	
P 0	10	75	85	n.s.
1	0	1	1	
2	0	1	1	
3	0	1	1	
Pathology				
Well	2	32	34	n.s.
Mod	8	37	45	
Other	0	9	9	

Microscopic staging of the tumors was performed according to "The General Rules for the Clinical and Pathological Study of Cancer of the Colon, Rectum and Anus."[13]

Table 3. Correlations between expression levels of mRNAs

Combination	Coefficient of correlation	*P* value
NT/DPD	0.3	0.002*
TP/DPD	0.76	5.26×10^{12}*
E2F1/DPD	0.141	0.168
DPD/TS	0.015	0.878
NT/TS	0.008	0.938
TP/TS	0.13	0.206
E2F1/TS	0.141	0.169
TP/NT	0.316	0.001*
E2F1/NT	0.054	0.599
E2F1/TP	0.08	0.438

* Statistically significant. TS, thymidylate synthetase mRNA; DPD, dihydropyrimidine dehydrogenase mRNA; TP, thymidine phosphorylase mRNA; NT, es-nucleoside transporter mRNA.

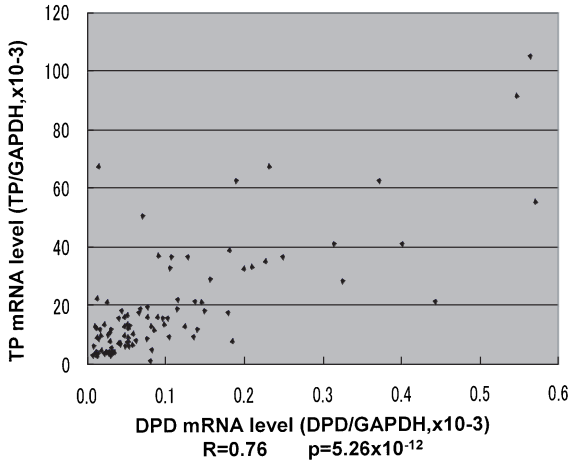

Fig. 1. Correlation between dihydropyrimidine dehydrogenase and thymidine phosphorylase mRNA expression. A high correlation was found, with a coefficient of correlation of 0.76.

Table 4. Correlations between chemosensitivity to 5-FU, and clinicopathological background factors and expression of mRNA

Factors	Coefficient of correlation	*P* value
Age	0.019	0.8617
Type	0.027	0.8046
t	0.031	0.7725
n	0.229	0.0316*
h	0.079	0.4635
p	0.074	0.4926
ly	0.031	0.7764
v	0.102	0.3467
Stage	0.129	0.2302
TS	0.16	0.1363
DPD	0.244	0.0229*
NT	0.257	0.0156*
TP	0.222	0.0376*
E2F1	0.246	0.0216*

* Statistically significant. TS, thymidylate synthetase mRNA; DPD, dihydropyrimidine dehydrogenase mRNA; TP, thymidine phosphorylase mRNA; NT, es-nucleoside transporter mRNA.

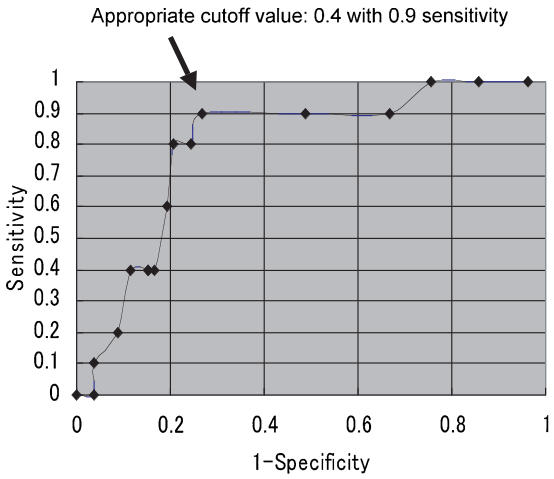

Fig. 2. Receiver operating characteristic curve of es-nucleoside transporter mRNA to predict the sensitivity to 5-fluorouracil of colon cancer specimens detected by MTT assay. There was an appropriate "shoulder," where the cutoff value was determined to be 0.4 with 90.0% sensitivity.

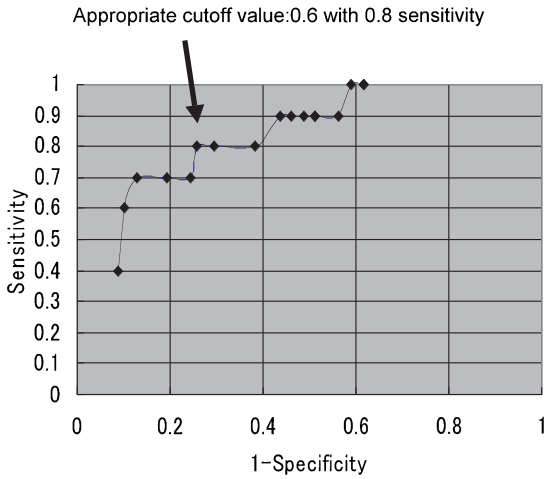

Fig. 3. Receiver operating characteristic curve of dihydropyrimidine dehydrogenase mRNA to predict the sensitivity to 5-fluorouracil of colon cancer specimens detected by MTT assay. There was an appropriate "shoulder," indicated by the arrow, where the cutoff value was determined to be 0.6 with 80.0% sensitivity.

mRNA expression and 5-FU sensitivity. Since the coefficient of correlation was less than 0.3 for each mRNA, the ROC curve was applied to confirm their usefulness as predictors for 5-FU sensitivity. Figs. 2, 3, and 4 show the ROC curves of NT, DPD, and TS mRNA, respectively, to predict the sensitivity to 5-FU detected by MTT assay. Although NT and DPD had an appropriate "shoulder" at the left and top part of the ROC curve, indicated by the arrow, TS mRNA did not show an appropriate point that would offer sufficient sensitivity and specificity. In addition, the ROC curves did not indicate the appropriate cutoff value for TP and E2F1 mRNAs, though they did correlate with 5-FU sensitivity in terms of coefficients of correlation (data not shown). After the cutoff values of NT mRNA and DPD mRNA were determined to be 0.4 with 90.0% sensitivity and 0.6 with 80.0% sensitivity from the shoulder of ROC curve, respectively, the specificity and the accuracy were calculated to predict the *in vitro* 5-FU sensitivity of colorectal cancer. We obtained values of 73.1% specificity and 75.0% accuracy for

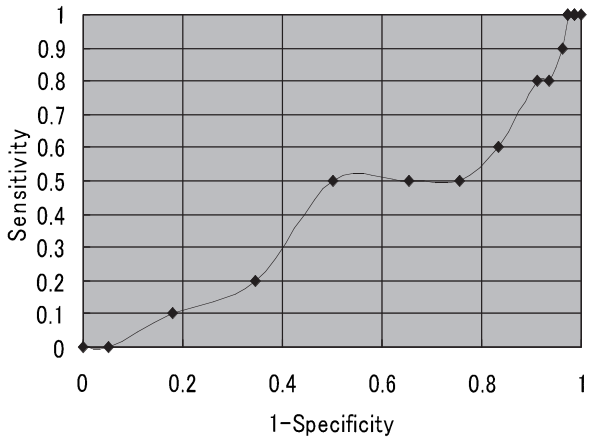

Fig. 4. Receiver operating characteristic curve of thymidylate synthetase mRNA to predict the sensitivity to 5-fluorouracil of colon cancer specimens detected by MTT assay. There was no appropriate "shoulder" in the ROC curve, and a suitable cutoff value could not be determined.

Table 5. Predictive value of es-nucleoside transporter and dihydropyrimidine dehydrogenase mRNA levels for *in vitro* 5-fluorouracil sensitivity of colorectal cancer

mRNA	Cutoff	Sensitivity (%)	Specificity (%)	Accuracy (%)
NT	0.4	90.0	73.1	75.0
DPD	0.6	80.0	74.4	76.1

After the appropriate cutoff value of mRNA was obtained from the shoulder of ROC curve, the predictive value was calculated. NT, es-nucleoside transporter mRNA; DPD, dihydropyrimidine dehydrogenase mRNA.

NT mRNA, and 74.4% specificity and 76.1% accuracy for DPD mRNA (Table 5).

Discussion

In the present study, we assessed the sensitivity of 88 surgical specimens of advanced colorectal cancer to 5-FU, and determined by measurements of the gene expression which mRNAs might predict their sensitivity to 5-FU. mRNAs analyzed included those of TS, DPD, TP, NT, and E2F1. TS is a target enzyme of 5-FU. An active metabolite, 5-fluorouridine monophosphate combines with TS when accompanied with a co-factor, 5,10-methylenetetrahydrofolate, to prevent *de novo* DNA synthesis. Several studies suggest that high TS expression will result in 5-FU resistance.[15–17] DPD is the first and rate-limiting enzyme in the catabolism of 5-FU. TP is the initial phosphorylating enzyme of 5-FU, which is anabolized to 5-fluorouridine monophosphate. This enzyme is also the key enzyme that acts upon doxifluridine, which is a metabolite of the newly investigated oral 5-FU prodrug, capecitabine, to produce 5-FU. NT is an es equilibrium membrane transporter of pyrimidines, and does not depend on a sodium pump.[18] E2F1 codes a representative transcriptional enzyme that transcribes some important messages, including TS and topoisomerase I, which may have a role in 5-FU resistance.

DPD and TP mRNA expressions were closely correlated with each other, with a coefficient of correlation of 0.76. These mRNAs code DPD and TP, which are not essential enzymes for DNA synthesis, but a catabolizing enzyme of pyrimidine for DPD and a salvage phosphorylating enzyme for TP. This result is consistent with the report of Mori *et al*.,[19] who found a close

correlation between DPD and TP expression levels detected by ELISA in 20 colorectal cancers (r=0.915 and P<0.0001). Although the reason for this close correlation between DPD and TP is unknown, these salvage pathways may work together to cover the requirement for *de novo* DNA synthesis.

When 5-FU sensitivity detected by MTT assay was compared with gene expression levels, DPD, NT, TP, and E2F1 mRNAs showed statistically significant correlations, while no correlation was observed between TS mRNA expression and 5-FU sensitivity. Several papers have reported that tumoral expression of TS mRNA has a statistically significant association with response to protracted infusion of 5-FU-based chemotherapy and survival in patients with gastric and disseminated colorectal cancer.[15–17] Since these clinical data were obtained with 5-FU and LV, the present result obtained with 5-FU alone may not be comparable. However, Etienne *et al*.[20] measured both TS and DPD activity in tumor biopsy specimens from 62 head and neck cancer patients before administration of 5-FU-based chemotherapy and reported that DPD activity was significantly related to 5-FU responsiveness, while no relationship was found between TS activity and response to 5-FU therapy. We have previously reported that in xenografts with high TS and low DPD levels, excess fluorodeoxyuridine monophosphate completely suppressed TS and the concentration of 5-FU in RNA increased gradually, resulting in both TS inhibition and RNA dysfunction.[21] The high concentration of substrate 5-FU in the medium may result in a less important role of TS in the resistance mechanism to 5-FU in the present study.

In addition, when the ROC curve was applied to confirm their usefulness as predictors for 5-FU sensitivity, only DPD and NT mRNAs appeared to be possible predictors of 5-FU sensitivity. Previously, we evaluated DPD enzymatic activity, and concluded that high DPD activity correlated with low chemosensitivity to 5-FU; we suggested that the activity of DPD represents a reliable indicator for the chemosensitivity of colon cancer to 5-FU.[22] Several studies using human tumor cell lines[23] and clinical samples[24] have indicated that 5-FU catabolism in tumor cells is probably a determining factor in 5-FU responsiveness. These results, combined with those of other reports,[25–27] suggest that tumor DPD activity and DPD mRNA level may be useful indicators for predicting the antitumor activity of 5-FU. The present result is comparable with those of the earlier studies, i.e., DPD is a possible predictor of 5-FU sensitivity, while TS is unrelated to 5-FU sensitivity detected by MTT assay.

Salvage of preformed nucleosides requires transport across the plasma membrane by sodium-dependent (concentrative) and sodium-independent (equilibrative) mechanisms. The assessed NT mRNA in the present study encodes nitrobenzylmercaptopurine riboside (NBMPR)-sensitive equilibrative nucleoside transporter (es or ENT1), which transports nucleoside from higher to lower concentration, and is inhibited by NBMPR. Since these transport systems are also involved in cellular uptake of nucleoside analogues, including gemcitabine and 5′-deoxy-5-fluorouridine, the cytotoxicity of these drugs might be influenced by NT. Rauchwerger *et al*. have observed a significant increase in NT levels in the presence of TS inhibitors, 5-FU or raltitrexed, pretreatment with which resulted in enhanced cytotoxicity of gemcitabine on two pancreatic cell lines.[28] In addition, Mackey *et al*. have demonstrated that deficiency in NT confers high-level resistance to gemcitabine toxicity *in vitro*, and the cytotoxic effect of 5′-deoxy-5-fluorouridine on MDA-MB-435 was reduced by NBMPR, suggesting that NT also mediates cellular uptake of nucleoside by breast cancer cells.[29] On the other hand, Boyer *et al*. have reported that potent inhibitors of NT, dipyridamole and NBMPR, showed synergistic cytotoxicity on 5-FU-resistant cell lines with NB1011, which competes with the natural substrate, deoxyuridine monophosphate, for binding to TS, suggesting that NT works as a resistance mechanism for 5-FU.[30] Thus, NT is thought to act as a resistance mechanism for 5-FU, while having a sensitizing function to nucleosides, including gemcitabine and 5′-deoxy-5-fluorouridine.

As regards 5-FU resistance, NT may transport active metabolites of 5-FU (5-fluorouridine and 5-fluorodeoxyuridine) out of tumor cells. On the other hand, the elevation of NT might also cause more cellular uptake of thymidine to rescue cells from thymine-less death by TS inhibition. Takechi and colleagues have investigated an mRNA differential display analysis to compare transcripts from the NUGC-3 human gastric tumor cell line and its 5-FU-resistant sub-line, in order to identify genes differentially expressed in association with resistance to 5-FU.[18] The 110 cDNA fragments differentially expressed in the analysis of either the wild-type or resistant cells were sequenced and subjected to a homology search, and 29 overexpressed and 22 underexpressed genes were identified in resistant lines as a result. The analysis revealed 20 overexpressed and 10 underexpressed genes in at least one of the three 5-FU-resistant cells as compared with the parent cells. Among them, P-glycoprotein, equilibrative nucleoside transporter 1 (NT), and methylenetetrahydrofolate dehydrogenase were highly expressed in two of the three 5-FU-resistant cell types.

Most 5-FU-based chemotherapy for colorectal cancer in clinical practice consists of a regimen with biochemical modulation, such as 5-FU/LV, and the third-generation oral 5-FU prodrugs, including orzel, S-1, and capecitabine, may eventually replace infusional 5-FU therapy. Although different predictive factors may be involved, including the amount of folate, enzymatic activities of cytidine deaminase, carboxyesterase, orotate phosphoribosyl transferase, and ribonucleotide reductase, the active metabolite of these prodrugs is presumably 5-FU, so 5-FU resistance mechanisms will still be important in the treatment of patients with colorectal cancer. In conclusion, the resistance mechanisms of 5-FU may include several changes in cellular function, but the catabolic rate-limiting DPD and membrane transporter NT are primarily involved in the clinically relevant resistance mechanisms. The alterations in expression of their genes should be useful predictive factors.

1. Mamounas E, Wieand S, Wolmark N, Bear HD, Atkins JN, Song K, Jones J, Rockette, H. Comparative efficacy of adjuvant chemotherapy in patients with Dukes' B versus Dukes' C colon cancer: results from four National Surgical Adjuvant Breast and Bowel Project Studies (C-01, C-02, C-03, C-04). *J Clin Oncol* 1999; **2**: 1349–55.
2. Wolmark N, Bryant J, Smith R, Grem J, Allegra C, Hyams D, Atkins J, Dimitrov N, Oishi R, Prager D, Fehrenbacher L, Romond E, Colangelo L, Fisher B. Adjuvant 5-fluorouracil and leucovorin with or without interferon alfa-2a in colon carcinoma: National Surgical Adjuvant Breast and Bowel Project protocol C-05. *J Natl Cancer Inst* 1998; **2**: 1810–6.
3. Dignam JJ, Colangelo L, Tian W, Jones J, Smith R, Wickerham DL, Wolmark N. Outcomes among African-Americans and Caucasians in colon cancer adjuvant therapy trials: findings from the National Surgical Adjuvant Breast and Bowel Project. *J Natl Cancer Inst* 1999; **91**: 1933–40.
4. Papamichael D. The use of thymidylate synthase inhibitors in the treatment of advanced colorectal cancer: current status. *Stem Cells* 2000; **18**: 166–75.
5. Advanced Colorectal Cancer Meta-analysis Project. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. *J Clin Oncol* 1992; **10**: 896–903.
6. Fujita K, Kubota T, Matsuzaki SW, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M. Further evidence for the value of the chemosensitivity test in deciding appropriate chemotherapy for advanced gastric cancer. *Anticancer Res* 1998; **18**: 1973–8.
7. Abe S, Kubota T, Matsuzaki SW, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M. Chemosensitivity test is useful in evaluating the appropriate adjuvant cancer chemotherapy for stage III non-scirrhous and scirrhous gastric cancers. *Anticancer Res* 1999; **19**: 4581–6.
8. Kubota T, Egawa T, Otani Y, Furukawa T, Saikawa Y, Yoshida M, Watanabe

M, Kitajima M. Cancer chemotherapy chemosensitivity testing is useful in evaluating the appropriate adjuvant cancer chemotherapy for stages III/IV gastric cancers without peritoneal dissemination. *Anticancer Res* 2003; **23**: 583–8.
9. Kabeshima Y, Kubota T, Watanabe M, Hasegawa H, Yamauchi T, Yorozuya K, Furukawa T, Otani Y, Kitajima M. Clinical usefulness of chemosensitivity testing in evaluating the optimal adjuvant chemotherapy for advanced colorectal cancer. *Anticancer Res* 2002; **22**: 3033–8.
10. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Kitajima M, Takechi T, Okabe H, Fukushima M. Dihydropyrimidine dehydrogenase activity and messenger RNA level may be related to the antitumor effect of 5-fluorouracil on human tumor xenografts in nude mice. *Clin Cancer Res* 1999; **5**: 883–9.
11. Ishikawa Y, Kubota T, Otani Y, Watanabe M, Teramoto T, Kumai K, Takechi T, Okabe H, Fukushima M, Kitajima M. Dihydropyrimidine dehydrogenase and messenger RNA levels in gastric cancer: possible predictor for sensitivity to 5-fluorouracil. *Jpn J Cancer Res* 2000; **91**: 105–12.
12. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**: 55–63.
13. Japanese Society for Cancer of the Colon and Rectum, editors. The general rules for the clinical and pathological study of cancer of the colon, rectum and anus. 6th ed. Tokyo: Kanehara Shuppan Co Ltd; 1998.
14. Fukushima M, Okabe H, Takechi T, Ichikawa W, Hirayama R. Induction of thymidine phosphorylase by interferon and taxanes occurs only in human cancer cells with low thymidine phosphorylase activity. *Cancer Lett* 2002; **187**: 103–10.
15. Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV, Leichman L. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* 1995; **6**: 1407–12.
16. Lenz HJ, Leichman CG, Danenberg KD, Danenberg PV, Groshen S, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Garcia Y, Li J, Leichman L. Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J Clin Oncol* 1996; **14**: 176–82.
17. Leichman CG, Lenz HJ, Leichman L, Danenberg K, Baranda J, Groshen S, Boswell W, Metzger R, Tan M, Danenberg PV. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. *J Clin Oncol* 1997; **15**: 3223–9.
18. Takechi T, Koizumi K, Tsujimoto H, Fukushima M. Screening of differentially expressed genes in 5-fluorouracil-resistant human gastrointestinal tumor cells. *Jpn J Cancer Res* 2001; **92**: 696–703.
19. Mori K, Hasegawa M, Nishida M, Toma H, Fukuda M, Kubota T, Nagasue N, Yamana H, Hirakawa YS, Chung K, Ikeda T, Takasaki K, Oka M, Kameyama M, Toi M, Fujii H, Kitamura M, Murai M, Sasaki H, Ozono S, Makuuchi H, Shimada Y, Onishi Y, Aoyagi S, Mizutani K, Ogawa M, Nakao A, Kinoshita H, Tono T, Imamoto H, Nakashima Y, Manabe T. Expression levels of thymidine phosphorylase and dihydropyrimidine dehydrogenase in various human tumor tissues. *Int J Oncol* 2000; **17**: 33–8.
20. Etienne M, Cheradame S, Fischel JL, Formento P, Dassonville O, Renee N, Schneider M, Thyss A, Demard F, Milano G. Response to fluorouracil therapy in cancer patients: the role of tumoral dihydropyrimidine dehydrogenase activity. *J Clin Oncol* 1995; **13**: 1663–70.
21. Inada T, Ichikawa A, Kubota T, Ogata Y, Moossa AR, Hoffman RM. 5-FU-induced apoptosis correlates with efficacy against human gastric and colon cancer xenografts in nude mice. *Anticancer Res* 1997; **17**: 1965–72.
22. Kobayashi N, Kubota T, Watanabe M, Otani Y, Teramoto T, Kitajima M. Pyrimidine nucleoside phosphorylase and dihydropyrimidine dehydrogenase indicate chemosensitivity of human colon cancer specimens to doxifluridine and 5-fluorouracil, respectively. *J Infect Chemother* 1999; **5**: 144–8.
23. Beck A, Etienne MC, Cheradame S, Fischel JL, Formento P, Renee N, Milano G. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. *Eur J Cancer* 1994; **30A**: 1517–22.
24. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. *Clin Cancer Res* 2000; **6**: 1322–7.
25. McLeod HL, Sludden J, Murray GI, Keenan RA, Davidson AI, Park K, Koruth M, Cassidy J. Characterization of dihydropyrimidine dehydrogenase in human colorectal tumors. *Br J Cancer* 1998; **77**: 461–5.
26. Johnston SJ, Ridge SA, Cassidy J, McLeod HL. Regulation of dihydropyrimidine dehydrogenase in colorectal cancer. *Clin Cancer Res* 1999; **5**: 2556–70.
27. Uetake H, Ichikawa W, Takechi T, Fukushima M, Nihei Z, Sugihara K. Relationship between intratumoral dihydropyrimidine dehydrogenase activity and gene expression in human colorectal cancer. *Clin Cancer Res* 1999; **5**: 2836–9.
28. Rauchwerger DR, Firby PS, Hedley DW, Moore MJ. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. *Cancer Res* 2000; **60**: 6075–9.
29. Mackey JR, Jennings LL, Clarke ML, Santos CL, Dabbagh L, Vsianska M, Koski SL, Coupland RW, Baldwin SA, Young JD, Cass CE. Immunohistochemical variation of human equilibrative nucleoside transporter 1 protein in primary breast cancers. *Clin Cancer Res* 2002; **8**: 110–6.
30. Boyer CR, Karjian PL, Wahl GM, Pegram M, Neuteboom ST. Nucleoside transport inhibitors, dipyridamole and *p*-nitrobenzylthioinosine, selectively potentiate the antitumor activity of NB1011. *Anticancer Drugs* 2002; **13**: 29–36.