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Decreased levels of UMP kinase as a mechanism of fluoropyrimidine resistance

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Abstract

5-Fluorouracil (5-FU) continues to be widely used for treatment of gastrointestinal cancers. Because many tumors show primary or acquired resistance, it is important to understand the molecular basis underlying the mechanism of resistance to 5-FU. In addition to its effect on thymidylate synthase inhibition and DNA synthesis, 5-FU may also influence RNA metabolism. Our previous studies revealed that colorectal cancer cells resistant to bolus 5-FU (HCT-8/4hFU) showed significantly decreased incorporation of the drug into RNA. Resistance to bolus 5-FU was associated with lower expression of UMP kinase (UMPK), an enzyme that plays an important role in the activation of 5-FU to 5-FUTP and its incorporation into RNA. Activities of other 5-FU–metabolizing enzymes (e.g., thymidine kinase, uridine phosphorylase, thymidine phosphorylase, and orotate phosphoribosyltransferase) remained unchanged between sensitive and resistant cell lines. Herein, we show that UMPK down-regulation in 5-FU–sensitive cells (HCT-8/P) induces resistance to bolus 5-FU treatment. Moreover, HCT-8/4hFU cells are even more cross-resistant to treatment with 5-fluorouridine, consistent with the current understanding of 5-fluorouridine as a RNA-directed drug. Importantly, colorectal cancer hepatic metastases isolated from patients clinically resistant to weekly bolus 5-FU/leucovorin treatment exhibited decreased mRNA expression of UMPK but not thymidylate synthase or dihydropyrimidine dehydrogenase compared with tumor samples of patients not previously exposed to 5-FU. Our findings provide new insights into the mechanisms of acquired resistance to 5-FU in colorectal cancer and implicate UMPK as an important mechanism of clinical resistance to pulse 5-FU treatment in some patients. [Mol Cancer Ther 2009;8(5):1037–44]

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the United States (1). 5-Fluorouracil (5-FU) has been the drug of choice in the clinic for the past several decades to treat patients diagnosed with this disease. In recent years, oxaliplatin and irinotecan have been used in combination with 5-FU and response rate and overall survival of patients with advanced disease has doubled. The response rate to 5-FU/leucovorin is 20% to 30% and intrinsic as well as acquired resistance to the drug is a major obstacle to successful treatment (2–5).

Two main schedules of 5-FU commonly used in the clinic are bolus short-term infusions given weekly with leucovorin and continuous infusions 24 hours to days. The mechanism of 5-FU action may differ depending on the dose and schedule of administration (Fig. 1A; ref. 6). Continuous infusion results in 5-FU anabolism mainly to FdUMP, which then acts as a tight binding inhibitor of thymidylate synthase (TS) and inhibits DNA synthesis (7). Bolus treatment favors drug anabolism to FUTP and its incorporation into RNA that interferes with normal pre-rRNA processing (8–10). As expected, resistance related to each mode of 5-FU administration may involve different factors and this

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is consistent with the observation that HCT-8 colon carcinoma cells resistant to bolus 5-FU treatment remain sensitive to continuous 5-FU exposure (11, 12). A common mechanism of clinical resistance to bolus and continuous 5-FU treatments is related to an increased level of TS and its inhibition (13, 14). No direct correlation of 5-FU incorporation into RNA and clinical response to bolus treatment with the drug has been reported. Our previous studies revealed that cells resistant to bolus 5-FU (HCT-8/4hFU) were similar to parental cell line with regard to the degree of *in situ* TS inhibition by 5-FU and duration of inhibition after 5-FU removal, whereas showed significantly decreased incorporation of the drug into RNA (15). It was of interest to broaden the molecular insight into this novel mechanism of 5-FU

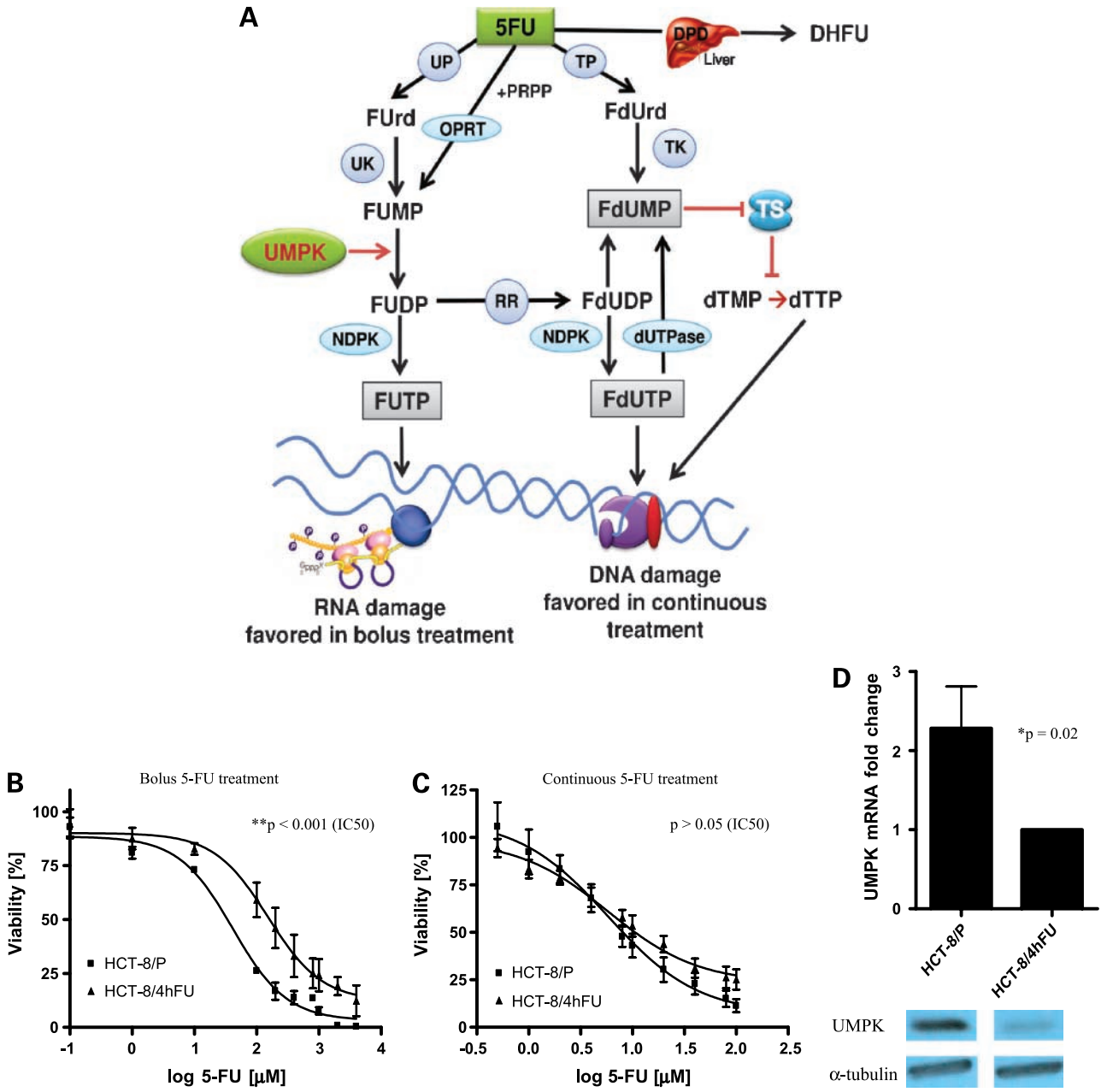

Figure 1. **A,** 5-FU has two different modes of action depending on dose and schedule of *in vitro* treatment. 5-FU activation to FUTP and its incorporation into RNA is favored in bolus treatment, and 5-FU activation to FdUMP that inhibits activity of TS using methylene tetrahydrofolate as a cofactor is favored in continuous exposure. Liver is the primary place of 5-FU degradation and requires DPD activity. *UK,* uridine kinase; *NDPK,* nucleoside diphosphate kinase; *RR,* ribonucleotide reductase; *dUTPase,* dUTP pyrophosphatase. **B,** HCT-8/4hFU cells are resistant to bolus 5-FU exposure. **C,** HCT-8/4hFU cells remain sensitive to continuous exposure to 5-FU. **D,** HCT-8/4hFU cells show decreased expression of UMPK mRNA (*top*) and protein levels (*bottom*). Experiments were done in triplicates and nonlinear regression with dose-response curve fitting and Student's *t* test comparing IC_{50} values or relative fold change was done to determine statistical significance.

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resistance found in HCT-8/4hFU cells and establish its contribution to observed clinical 5-FU resistance.

UMP/CMP kinase (UMPK) catalyzes the transfer of the phosphate group to UMP, CMP, and dCMP using ATP as a cofactor in the presence of magnesium (16). This enzyme is crucial for *de novo* and salvage synthesis of pyrimidine nucleotides and no other enzyme with the same substrate specificity as UMPK has been identified thus far (17). Besides its biological function, UMPK plays a very important role in the activation of nucleoside analogues used as anticancer or antiviral drugs, including 5-FU, zebularine, 1-β-D-arabinofuranosylcytosine, 2′,2′-difluorodeoxycytidine (gemcitabine), β-D-2′,3′-dideoxycytidine, β-L-2′,3′-dideoxy-3′-thiacytidine, and 2′,3′-deoxy-3′-azidothymidine (18). Importantly, UMPK is thought to be a rate-limiting enzyme in the conversion of nucleosides and its analogues to triphosphates in the cell (19). Despite the important function carried out by UMPK, little is known about the regulation of this enzyme. The cDNA for human UMPK was cloned recently based on its homology with pig UMPK (20, 21).

Materials and Methods

In vitro Colorectal Cancer Model

HCT-8 cells (human colon cancer cells) were maintained in RPMI 1640 supplemented with 10% horse serum, 1 mmol/L sodium pyruvate, and antibiotics. The cells were cultured under standard cell culture conditions (i.e., humidified atmosphere of 95% air and 5% CO_2 at 37°C). Bolus 5-FU–resistant colorectal cancer cell lines were developed in a way that most closely mimics the clinical situation under which this resistance occurs, as described (15, 22). Briefly, cells were exposed to 10^{-4} mol/L 5-FU for 4 h at 14- to 21-d intervals. Between drug exposures, cells were allowed to recover in drug-free culture medium. Drug-resistant colonies were expanded as cell lines and designated HCT-8/4hFU. Cell line designated YALE/HCT-8 is a bolus 5-FU–resistant cell line independently developed at Yale University using similar protocol and used as an additional control in some of our experiments (22).

UMPK Down-Regulation

Gene knockdown was done using small interfering RNA (siRNA) technology. siRNAs were designed for two different exons of the *UMPK* gene and synthesized by Ambion. HCT-8/P cells were transiently transfected with siRNA using Oligofectamine reagent (Invitrogen) and a standard protocol. Briefly, cells were seeded at 0.25×10^6 in cell culture medium without antibiotics the day before transfection in six-well plates. Transfection was done in Opti-MEM serum-free medium (Invitrogen) and fresh growth medium was added 12 h after transfection. Final concentrations of siRNAs tested were 20 and 40 nmol/L. Scrambled siRNA was used as a negative control (Ambion). Cellular levels of UMPK mRNA and protein were analyzed by quantitative reverse transcription-PCR (RT-PCR) and Western blotting 24, 48, and 72 h following the transfection to find the dose of siRNA and time after transfection that resulted in effective knocking down of UMPK. Subsequently, siRNA-transfected cells were assayed for their sensitivity to bolus and continuous exposure to 5-FU. Cells were subcultured 48 h after transfection.

5-FU/5-Fluorouridine Bolus Exposure

To mimic the clinical use of bolus 5-FU treatment in the *in vitro* conditions, exponentially growing cells were exposed to 5-FU (0.1–500 μmol/L) or 5-fluorouridine (5-FUR; 0.001–100 μmol/L) for 4 h (Sigma). Then, drug-containing medium was removed, cells were washed once with PBS, and drug-free growth medium was added. Following drug exposure, cells were allowed to grow for 4 d before doing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay.

5-FU Continuous Exposure

To model the other clinically relevant use of 5-FU (i.e., continuous infusion), exponentially growing cells were exposed to 5-FU (0.001–100 μmol/L) for 4 d. Cytotoxicity was determined by the MTS assay.

MTS Assay

Sensitivity of cells to 5-FU and 5-FUR was measured using the MTS assay (Promega). Briefly, 2,000 cells were plated in the 96-well plates (Corning) the day before addition of the drug. Dilutions of 5-FU (Sigma) and 5-FUR were prepared fresh in RPMI 1640 growth medium. The manufacturer's protocol was followed for the preparation and use of the MTS reagent.

Colorectal Cancer Patient Samples

A total of 29 colorectal tumor liver metastases were obtained from 29 different colorectal cancer patients. Nineteen patients were previously treated with bolus 5-FU weekly, plus leucovorin, and either did not respond or had responded and then relapsed. The additional 10 patients included in this study were untreated with 5-FU and presented with hepatic metastasis of colorectal cancer. The tumors were obtained at the time of surgery before resection of hepatic metastasis or pump placement for hepatic infusion therapy. All patients provided written informed consent. Diagnosis was made in the Department of Pathology, Memorial Hospital (New York, NY) using a portion of the tumor specimen. Ultraspec reagent (Biotecx Labs) was added to a viable portion of the tumor sample within minutes after removal, and the tumor sample was flash frozen in liquid nitrogen until the time of analysis. At the time of analysis, tumors were ground to a powder in liquid nitrogen and both RNA and protein (only in case of large enough tumors) were extracted and quantitative RT-PCR and Western blotting for UMPK protein level were done. mRNA levels for UMPK, TS, and dihydropyrimidine dehydrogenase (DPD) were assayed as described below. RNA was reverse transcribed using random primers (Invitrogen), and the product was used as template for subsequent PCR using Universal PCR Master Mix (Applied Biosystems) and LightCycler System (Roche). The relative UMPK, TS, and DPD mRNA levels (y) were assayed and calculated according to the formula $y = (100 - x)$, where $x = [(Ct_{UMPK} - Ct_{\beta\text{-actin}})/Ct_{\beta\text{-actin}}] * 100$. The sequences of the primers were as follows:

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UMPK, 5′-TTGACCCGTCTCCATCGG-3′ (forward), 5′-TGCCTCCTGACCCCTCCT-3′ (reverse), and 5′-FAM-CCCCAGCCCCTATCTCCAAGAGACA-3′-TAMRA (probe); β-actin, 5′-TGAGCGCGGCTACAGCTT-3′ (forward), 5′-TCCTTAATGTCACGCACGATTT-3′ (reverse), and 5′-FAM-ACCACCACGGCCGAGCGG-3′-TAMRA (probe); TS, 5′-GGCCTCGGTGTGCCTTT-3′ (forward), 5′-GATGTGCGCAATCATGTACGT-3′ (reverse), and 5′-FAM-AACATCGCCAGCTACGCCCTGC-3′-TAMRA (probe); and DPD, 5′-GGTATGCAGTGCCATTCAGA-3′ (forward), 5′-TGGTTTCCCTTTCTGGTGAC-3′ (reverse), and 5′-FAM-TGCACTGGCCTCAAAGCCCTG-3′-TAMRA (probe).

UMPK Activity Assay

Cells from both parental and 5-FU bolus-resistant HCT-8 lines were harvested and snap frozen in liquid nitrogen until used. Pellets were thawed in extraction buffer containing 20 mmol/L potassium phosphate (pH 8.0), 1 mmol/L $MgCl_2$, 2 mmol/L DTT, 7.2 mg/mL aprotinin, 1 mg/mL pepstatin A, 1 mg/mL leupeptin, and 100 mmol/L phenylmethylsulfonyl fluoride. Cells were sonicated on ice for three 15-s bursts. The resulting homogenate was centrifuged at 100,000 × *g* for 1 h at 4°C and the cell-free supernatant was stored at −70°C until enzyme assays were done. UMPK activity was assayed in 1 mL reaction mixtures containing 100 mmol/L Tris-HCl (pH 8.0), 500 mmol/L EDTA, 100 mmol/L KCl, 25 mmol/L $MgCl_2$, 2 mmol/L ATP, 2.5 mmol/L phosphoenolpyruvate, 200 mmol/L NADH, 50 units/mL pyruvate kinase, and 250 units/mL lactate dehydrogenase (23). The protein extract (10 mL) was added to the reaction mixture and incubated at 37°C for 10 min. UMPK activity was determined by adding UMP (ranging from 0.04 to 0.20 mmol/L) or FUMP (0.25 and 1.25 mmol/L) and measuring the decrease in absorbance of NADH at 340 nm for 10 min using a Gilford 2400 spectrophotometer. Concentrations of UMP and FUMP were determined by measuring the absorbance at 262 nm (molar extinction coefficient, 10.0) or at 265 nm (molar extinction coefficient, 8.1), respectively. A molar extinction coefficient of 12.44 was used for the calculation of UMPK activity, which was expressed as mmol substrate converted per mg of protein per minute. The activity was linear with respect to time at all substrate concentrations used. A Lineweaver-Burk plot was used to determine the K_m and V_{max} of UMPK enzyme from the different cell lines. The assay was done thrice in triplicate with UMP as substrate and twice in triplicate with FUMP as substrate. Other enzyme activities measured included TS, thymidine kinase (TK), uridine phosphorylase (UP), thymidine phosphorylase (TP), and orotate phosphoribosyltransferase (OPRTase). These were carried out by previously described methods (15, 22).

Western Blotting

Cells (1–5 × 10^6) were recovered by trypsin treatment, washed twice with PBS, and kept frozen in −80°C until used. Cell pellets or powdered patient tumors were thawed and resuspended in 0.5 mL of radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 3 mmol/L sodium fluoride, 4 mmol/L DTT] supplemented with protease inhibitors. Samples were sonicated (2 × 20 s, 60% power) on ice and centrifuged for 20 min at 20,000 rpm to remove insoluble cell debris. Lysate (50 mg) was loaded onto 10% SDS-PAGE gel and, after transfer to nitrocellulose, probed with anti-UMPK polyclonal antibody (1:1,000) for 2 h (gift of Dr. Y-C. Cheng) and secondary ant-rabbit antibody (1:5,000) for 1 h (Sigma) or anti–α-tubulin (1:10,000) for 1 h (Sigma) and anti-mouse antibody (1:2,000) for 0.5 h (Santa Cruz Biotechnology) as a control.

Quantitative RT-PCR

UMPK mRNA levels in HCT-8 cells were done as described above. Total RNA was extracted from 1 × 10^6 to 5 × 10^6 cells using Trizol reagent (Invitrogen) and run on 1% formaldehyde-agarose gel to assess the quality of the RNA. Gene-specific primers and fluorescent-labeled probe (FAM and TAMRA) were designed using Primer Express Software from ABI (Applied Biosystems). RNA (50 ng) was amplified in one-step RT-PCR (Applied Biosystems) using Taqman Real-Time PCR machine (7000 SDS). 18S rRNA (FAM; Applied Biosystems) or β-actin (FAM-TAMRA) was used to normalize the results. ΔΔCt method was used to calculate relative fold change in mRNA level (24). The sequence of the UMPK primer is as follows: forward, 5′-AAGAAG-GAAAGATTGTACCAGTTGAGA-3′; reverse, 5′-GGAAAC-CCATCAATCAAGAATTTATT-3′; probe, 5′-FAM-AGAGG-GAAATGGATCAGACAATGGCTGC-TAMRA-3′.

Statistical Analysis

GraphPad Prism 4 was used for statistical analysis and statistical significance was assayed using Student's *t* test. Gels were quantitated using a ChemiDoc XRS machine and Quantity One one-dimensional analysis software (Bio-Rad).

Results

Decreased UMPK Level Mediates Resistance to Bolus 5-FU

To study molecular mechanisms underlying resistance to pulse 5-FU, we repeated previous studies of Sobrero et al. (11) and exposed the colorectal cancer cell line HCT-8 to repeated fixed doses of 5-FU (see Materials and Methods for more details). Resulting bolus 5-FU–resistant clones were denoted as HCT-8/4hFU and the parental cell line

Table 1. Specific activity of UMPK from parental and bolus 5-FU–resistant cell lines

Substrate	Specific activity*	
	UMP	FUMP
HCT-8/P	0.14 ± 0.01	0.21
HCT-8/4hFU	0.09 ± 0.02	0.17
HCT-8R/YALE	0.09 ± 0.01	0.19

NOTE: Measurements ± SD shown above were recorded in the presence of 0.2 mmol/L UMP (biological triplicates) or 1.25 mmol/L FUMP as the substrate (biological duplicates). Samples were measured each time in triplicates.
*Specific activity of UMPK is expressed as μmol/mg/min.

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Table 2. Specific activities of other 5-FU–metabolizing enzymes

	HCT-8/P	HCT-8/4hFU
UMPK	8,400 ± 600	5,400 ± 1,200
TP	4.8 ± 0.2	5.1 ± 2.8
TK	3.3 ± 1.9	3.1 ± 2.4
TS	24.4 ± 6.9	15.5 ± 5.8
UP	3.8 ± 2.5	4.9 ± 2.8
UK	31.9 ± 9.8	25.7 ± 9.3
OPRTase	10.5 ± 3.7	12.4 ± 2.0

NOTE: Specific activities of each enzyme are expressed as nmol/mg/h. Samples were measured in triplicates and three independent experiments were done.

as HCT-8/P. Cell viability assays confirmed that HCT-8/4hFU cells are 3- to 4-fold more resistant to the treatment with bolus 5-FU but equally sensitive to continuous exposure to 5-FU (Fig. 1B and C). Lack of cross-resistance between the two treatment schedules supports the notion that resistance to each 5-FU treatment involves different mechanisms as depicted in Fig. 1A. Our previous studies indicated that resistance to bolus 5-FU was associated with a lower incorporation of the drug into RNA without changes in TS inhibition or drug incorporation into DNA (15). To uncover an underlying mechanism of decreased 5-FU incorporation into RNA, enzymatic activities of the major enzymes participating in 5-FU anabolism and its activation to 5-FUTP were assayed (i.e., TP, TK, TS, UP, UK, and UMPK). Activity of UMPK was found to be decreased in HCT-8/4hFU and HCT-8/YALE compared with the parental HCT-8/P cell line ($P < 0.05$) when measured in the presence of UMP as a substrate (Table 1). Moreover, kinetic analysis of UMPK revealed no change in the K_m value of the enzyme between the resistant and the parental cell lines when either UMP ($K_m = 0.3$) or FUMP ($K_m = 2.5$) was used as a substrate. Activities of all the other 5-FU–metabolizing enzymes assayed were not significantly altered between the two cell lines ($P > 0.05$; Table 2). This result suggested that decrease in the UMPK activity is due to a lower abundance of the enzyme rather than inactivation of UMPK enzyme due to mutation. Indeed, further analysis of mRNA and protein levels of UMPK showed a decrease of 2- to 3-fold in resistant cells compared with HCT-8/P (Fig. 1D). Please note that observed decrease in UMPK protein level in HCT-8/4hFU cells was not due to protein degradation. This result likely explains the reason for the previously reported lower incorporation of 5-FU into RNA in bolus-resistant cells. The HCT-8R/YALE cell is another bolus 5-FU–resistant cell line developed independently in the laboratory of Dr. G. Pizzorno at Yale University using a protocol based on intermittent dosing to mimic changes in the 5-FU plasma concentration following drug administration (22). Of interest, decreased levels of UMPK were also found in this resistant cell line (Supplementary Fig. S1).[7]

UMPK Down-Regulation in HCT-8/P Cells Confers Resistance to Bolus 5-FU

To further confirm the role of UMPK in development of resistance to 5-FU, mRNA levels were knocked down in 5-FU–sensitive cells (HCT-8/P) using siRNA. Two different siRNAs were designed, targeting exon 1 (siRNA#1) and exon 3 (siRNA#3) of the *UMPK* gene. Following the standardization of transfection efficiency, we selected the 72-hour time after transfection and siRNA#3 or a combination of

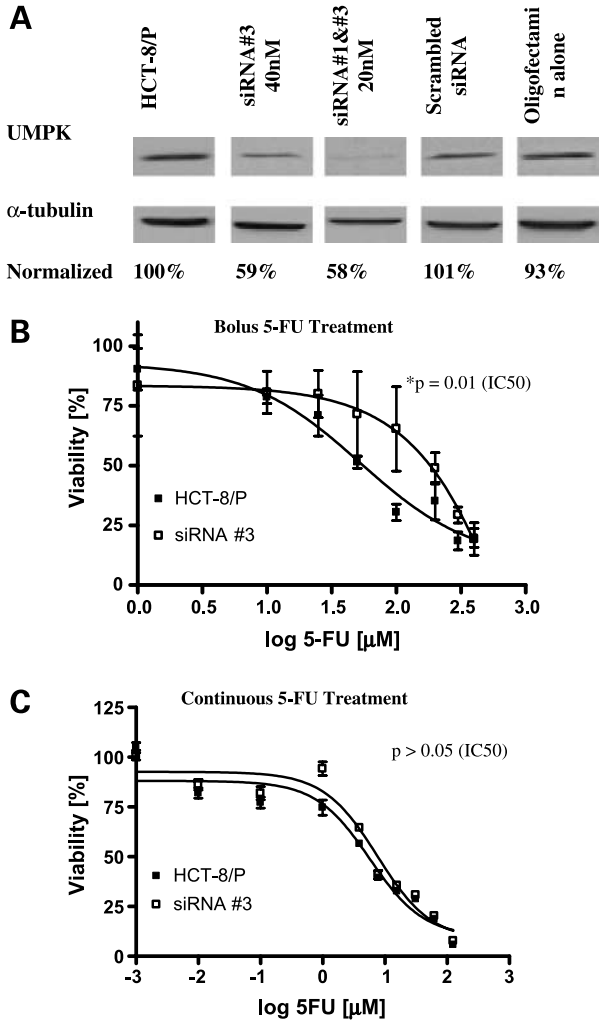

Figure 2. Down-regulation of UMPK in HCT-8/P cells induces resistance to bolus 5-FU but not continuous exposure. **A,** efficiency of siRNA-mediated down-regulation of UMPK protein compared with scrambled siRNA and Oligofectamine treatment. **B,** bolus 5-FU dose-response curves of HCT-8/P cells transfected with siRNA designed for *UMPK* gene (siRNA#3 at 40 nmol/L) compared with untransfected cells. **C,** continuous 5-FU dose-response curves of HCT-8/P cells transfected with siRNA designed for *UMPK* gene (siRNA#3 at 40 nmol/L) compared with untransfected cells. Experiments were done in triplicates and nonlinear regression with dose-response curve fitting and Student's *t* test comparing IC_{50} values was done to determine statistical significance.

[7] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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siRNA#1 and siRNA#3 that resulted in a 50% decrease in UMPK protein (Fig. 2A) and mRNA levels (data not shown). Cytotoxicity assays revealed that down-regulation of UMPK conferred resistance to bolus 5-FU treatment (Fig. 2B) but not to continuous 5-FU exposure (Fig. 2C). Moreover, scrambled siRNA or Oligofectamine alone did not influence sensitivity of HCT-8/P cells to bolus 5-FU (Supplementary Fig. S2A).[7] These data further support the association of decreased levels of UMPK with acquired resistance to bolus 5-FU.

HCT-8/4hFU Cells Display Cross-Resistance to 5-FUR

5-FUR is a 1-β-D-ribofuranoside analogue of 5-FU and its cytotoxic action is mainly RNA directed (25). Similarly to bolus 5-FU, it requires activity of UMPK to be activated to 5-FUTP that is incorporated into RNA. As expected, and confirming the RNA mechanism of pulse treatment with 5-FU, HCT-8/4hFU cells were even more resistant to 5-FUR (Fig. 3A). A 10-fold increase in the 5-FUR IC_{50} value of HCT-8/4hFU cells was observed compared with HCT-8/P cells. Moreover, down-regulation of UMPK in HCT-8/P cells using siRNA#3 and combination of siRNA#1 and siRNA#3 (Fig. 3B) but not scrambled siRNA (Supplementary Fig. S2B)[7] induced resistance to 5-FUR. These results are consistent with the premise that bolus 5-FU

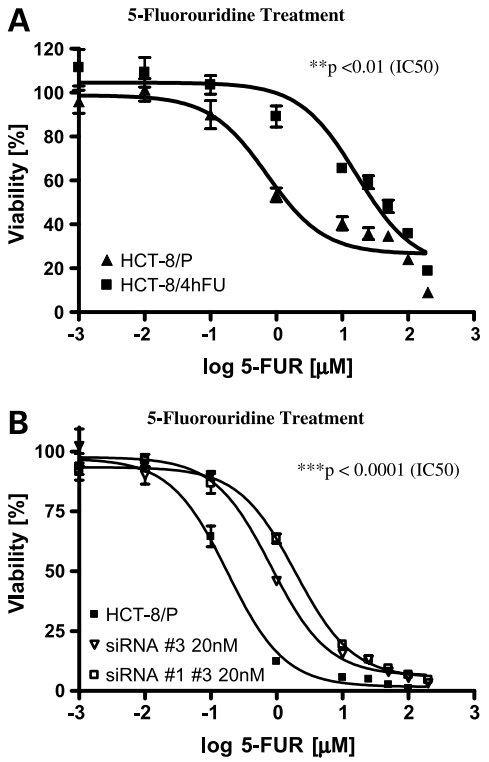

Figure 3. Bolus 5-FU–resistant cells are cross-resistance to 5-FUR. **A,** 5-FUR dose-response curves of HCT-8/P and HCT-8/4hFU cells. **B,** 5-FUR dose-response curves of HCT-8/P cells transfected with siRNA designed for *UMPK* gene (siRNA#3 at 20 nmol/L) or combination of siRNA#1 and siRNA#3 at 20 nmol/L each. Experiments were done in triplicates and nonlinear regression with dose-response curve fitting and Student's *t* test comparing IC_{50} values was done to determine statistical significance.

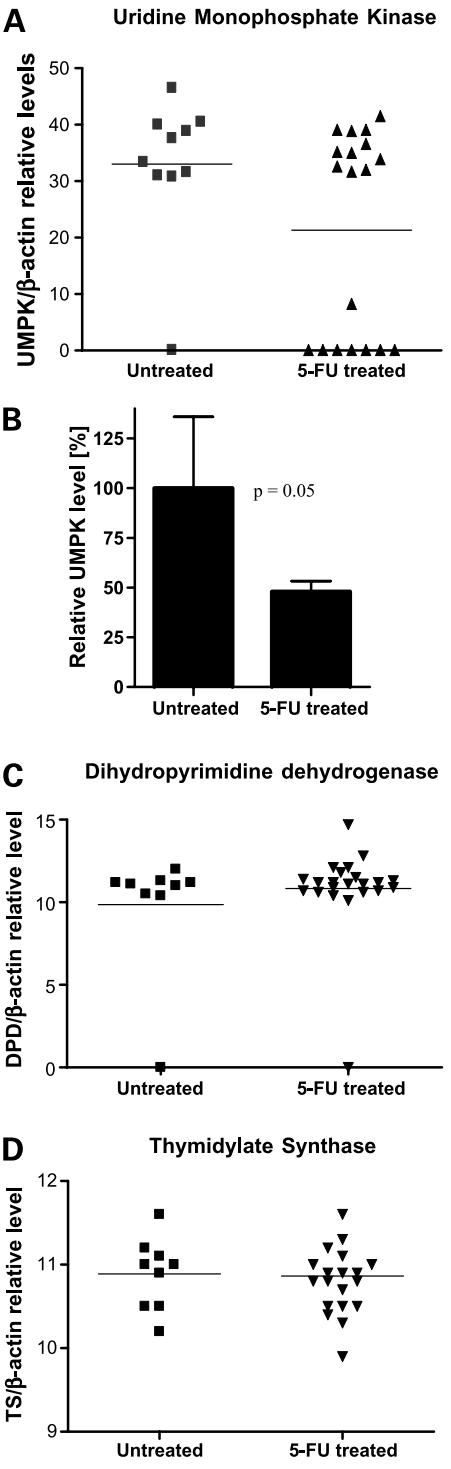

Figure 4. Liver metastasis from patients previously exposed to bolus 5-FU showed higher incidence of decreased UMPK but not TS or DPD levels. **A,** UMPK mRNA level was quantitated in 10 samples of patients not previously exposed to bolus 5-FU and 19 samples of 5-FU–treated patients. Expression was normalized to β-actin as described in Materials and Methods. **B,** UMPK protein level in samples of 5-FU–treated patients (nine samples) and 5-FU–untreated patients (four samples). UMPK protein level was normalized to α-tubulin, averaged, and expressed as relative percentage. *Columns,* mean; *bars,* SD. **C,** DPD mRNA level in patient samples. **D,** TS mRNA level in patient samples.

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administration is RNA directed and implicates UMPK in the development of resistance to bolus 5-FU.

Analysis of Colorectal Cancer Patient Samples

We analyzed 29 metastatic colorectal tumor samples: 10 were from patients that had not received any prior 5-FU treatment, whereas the other 19 had received systemic 5-FU treatment weekly with leucovorin (Roswell Park Regimen). Seven of 19 previously treated patients had undetectable levels of UMPK mRNA and 1 sample had low levels of expression of UMPK. In the group of untreated patients, only 1 of 10 tumors had no detectable UMPK mRNA (Fig. 4A). Western blotting analysis on 13 samples for which lysates were available (9 of 5-FU–treated patients and 4 of untreated patients) showed that at the protein level UMPK expression in 5-FU–treated samples was on average 2-fold lower compared with the tumor samples from patients not previously treated with 5-FU (Fig. 4B). Analysis of expression of two other enzymes commonly linked to 5-FU resistance (i.e., DPD and TS) between the two groups of patients did not show significant changes (Fig. 4C and D). Hence, ~40% of patients that were previously treated with 5-FU may have failed the treatment due to acquired resistance mediated by decreased expression of UMPK.

Discussion

Several predictive biomarkers for 5-FU response have been identified in colorectal cancer. However, their clinical utility still remains controversial. A high level of TS and its promoter polymorphism has been associated with decreased responsiveness, particularly to infusional 5-FU (26). A low expression of *TP* gene and high expression of DPD also have been correlated with resistance to 5-FU (27). p53 status has been studied as a predictor of responsiveness to cancer chemotherapy but conflicting results have been reported relating this tumor suppressor to response to 5-FU (28). Analysis of colorectal cancer hepatic metastasis of patients previously treated with 5-FU showed a high frequency of low or undetectable expression of UMPK. To the best of our knowledge, this is the first report that directly links lower expression of UMPK in patients to the development of 5-FU resistance. Interestingly, recently published studies using whole-genome profiling in search of 5-FU resistance-mediating genes suggested *UMPK* as candidate gene. Analysis of gene expression profiles of various cell lines resistant to 5-FU showed a 3-fold decrease in UMPK mRNA (29). Using a similar approach, Schmidt et al. (30) showed UMPK down-regulation as one of the descriptors of the intermediate resistance phenotype, metastasis potential stage, but not primary resistance to 5-FU.

Genomic instability is one of the characteristics of colorectal cancer and the *UMPK* gene, mapped on chromosome 1p32, is often lost in the deletion of short arm of chromosome 1. However, data suggest that UMPK gene down-regulation may occur before deletion of this part of the chromosome (31). Therefore, low UMPK expression may also be a potential biomarker for a more advanced or malignant tumor phenotype that is less responsive to chemotherapy.

Our data indicate that UMPK mediates resistance only to bolus 5-FU but not continuous 5-FU. As we have not studied resistance in patients treated with continuous infusions of 5-FU, additional clinical studies are needed to validate that this resistance mechanism is restricted to patients receiving bolus 5-FU treatments. It is possible that decreased expression of UMPK leads to decreased formation of FdUMP and decreased or transient inhibition of TS, especially under low folate concentrations. Because cell lines are cultured under high nonphysiologic folate concentrations, the lack of differences in the *in situ* TS inhibition might not reflect the case in human tumors.

Ongoing studies are directed toward explaining the lower expression of UMPK in HCT-8/4hFU cells. Pizzorno and Handschumacher (22) showed that 5-FU–resistant colorectal cancer cell lines generated using clinically modeled regimens may revert to their original sensitivity with time. This suggests that resistance may be mediated by reversible epigenetic changes rather than genetic mutations. Indeed, our studies, the focus of the concurrent article, showed that UMPK down-regulation occurs at the transcriptional level and is fully reversible *in vitro* as well as in mouse xenograft tumors by administration of epigenetic modifiers (32). Understanding of the molecular reasons underlying 5-FU resistance is crucial for predicting as well as overcoming resistance by development of strategies that increase its anticancer activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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