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Epigenetic reversal of acquired resistance to 5-fluorouracil treatment

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Abstract

Acquired and intrinsic resistance still remains a limitation to the clinical use of 5-fluorouracil (5-FU). The contribution of epigenetic changes to the development of drug resistance remains to be elucidated. Several genes that are hypermethylated and silenced have been identified in colorectal cancer. Based on the findings described in the accompanying article, we hypothesized that acquired resistance to "pulse" 5-FU has an epigenetic origin and might be reversed. Here, we present a novel therapeutic approach to circumvent clinical resistance to bolus 5-FU, that is, treatment of bolus 5-FU-resistant colorectal cancer cells with low-dose 5-azadeoxycytidine (DAC), an inhibitor of DNA hypermethylation, restored sensitivity to 5-FU as well as 5-fluorouridine. Moreover, treatment of nude mice bearing a 5-FU-resistant tumor, characterized by decreased levels of UMP kinase (UMPCK), with DAC overcame resistance to bolus 5-FU. DAC-mediated restoration of 5-FU sensitivity was associated with increases in UMPCK levels. An increase in UMPCK protein and mRNA levels following treatment with low-dose DAC was observed in cultured bolus 5-FU-resistant colorectal

cancer cells (HCT-8) and in mice bearing these tumors. We conclude that DAC-mediated restoration of sensitivity to bolus 5-FU is mediated at least in part by increased UMPCK levels and clinical resistance to 5-FU due to decreased UMPCK in colorectal cancer may be overcome by including methylation inhibitors such as DAC. [Mol Cancer Ther 2009;8(5):1045–54]

Introduction

Epigenetic events contribute significantly to the development and progression of cancer through inactivation of tumor suppressors, DNA repair genes, and growth-regulatory microRNAs (1–3). These alterations have been proposed to precede or even facilitate genetic mutations or genomic instability and chromosome translocations that have long been associated with carcinogenesis (4, 5). It is of importance to identify genes or chromosomal regions, the epigenetic alterations of which foster clonal expansion and accumulation of additional genetic modifications. Epigenetic alterations are known to involve either methylation of DNA and/or resetting the complex code of histones that in turn leads to changes in chromatin structure affecting transcription. The molecular determinants of changes in chromatin structure within tumor cells are only beginning to be elucidated. Best understood is the role of promoter DNA methylation in silencing of tumor suppressor genes (6–11). A substantial number of genes with promoter hypermethylation have been identified in colorectal cancer (12–17).

Increasing evidence supports the notion that epigenetic changes also play a driving force behind the acquisition of drug resistance (18). Multiple changes in the methylation of CpG islands have been observed following drug selection in ovarian cancer (19). Methylation of the proapoptotic factors Apaf-1 and caspase-8 has been reported to be associated with resistance to doxorubicin, etoposide, and cisplatin (20, 21). Interestingly, the MDR1 promoter was found to be progressively demethylated during the course of chemotherapy of acute myeloid leukemia and was correlated with overexpression of P-glycoprotein (22). Analysis of methylation of the DNA mismatch repair gene human MLH1, revealed therapy-related acquisition of methylation of this gene in 25% of patients and this was correlated with poor overall survival for ovarian cancer patients (23). MLH1 promoter methylation has also been shown to be associated with loss of DNA mismatch repair in colon cancer and resistance to

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5-fluorouracil (5-FU; ref. 14). Epigenetic-driven changes in gene expression provide a faster and still heritable means by which tumor cells can adapt to stress in the environment that cytotoxic drug therapy induces.

It remains to be established whether inhibition of epigenetic changes occurring following cytotoxic drug exposure will prevent development of subsequent genetic changes that lead to high-grade resistance and/or acquisition of a metastatic phenotype. 5-Azadeoxycytidine (DAC), a hypermethylation inhibitor, was recently approved for the treatment of patients with the myelodysplastic syndrome (24). When used at lower doses, DAC reactivates tumor suppressor genes and tumor antigens that are silent due to methylation, without significant cytotoxicity (25). Despite this observation, to date, only a limited number of clinical trials exploring the potential of DAC alone or in combination with existing chemotherapy in the treatment of solid tumors have been reported. Plumb et al. showed that DAC-mediated reactivation of human MLH1 expression reverses drug resistance in human tumor xenografts (26). Subsequently, a clinical trial has been initiated exploring the combination of DAC and carboplatin in solid tumors (27).

Understanding the molecular basis for acquired resistance to drug treatment offers a rationale for therapeutic intervention that may result in better treatment outcomes. Based on the above arguments, we hypothesized that 5-FU resistance of colorectal cancer due to decreased UMP kinase (UMP5K) activity secondary to bolus exposures to the drug would be reversed using DAC. We tested this hypothesis *in vitro* and in a colorectal cancer xenograft model.

Materials and Methods

In vitro Colorectal Cancer Model

Human intestinal adenocarcinoma cells (HCT-8) were maintained in RPMI 1640 supplemented with 10% horse serum, 1 mmol/L sodium pyruvate, and antibiotics. The cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ 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 previously (28, 29). Briefly, cells were exposed to repeated doses of 10⁻³ mol/L 5-FU for 4 h. Between drug exposures, cells were allowed to recover in drug-free culture medium. Drug-resistant colonies were expanded as cell lines and are designated HCT-8/4hFU.

Drug Treatment

DAC (Sigma) was dissolved in DMSO and stored on ice until used. Exponentially growing cells were exposed to various low concentrations of the drug for 24 h and then immediately treated with 5-FU or 5-fluorouridine (5-FUR) or harvested for DNA, RNA, or protein analysis. 5-FU and 5-FUR (Sigma) solutions were prepared fresh before each use in culture medium. Cells were exposed to 5-FU (0.1-500 μmol/L) or 5-FUR (0.001-100 μmol/L) for 4 h. Then, drug-containing medium was removed, cells were washed once with PBS, and drug-free growth medium was added and cells were cultured for 4 days before performing 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

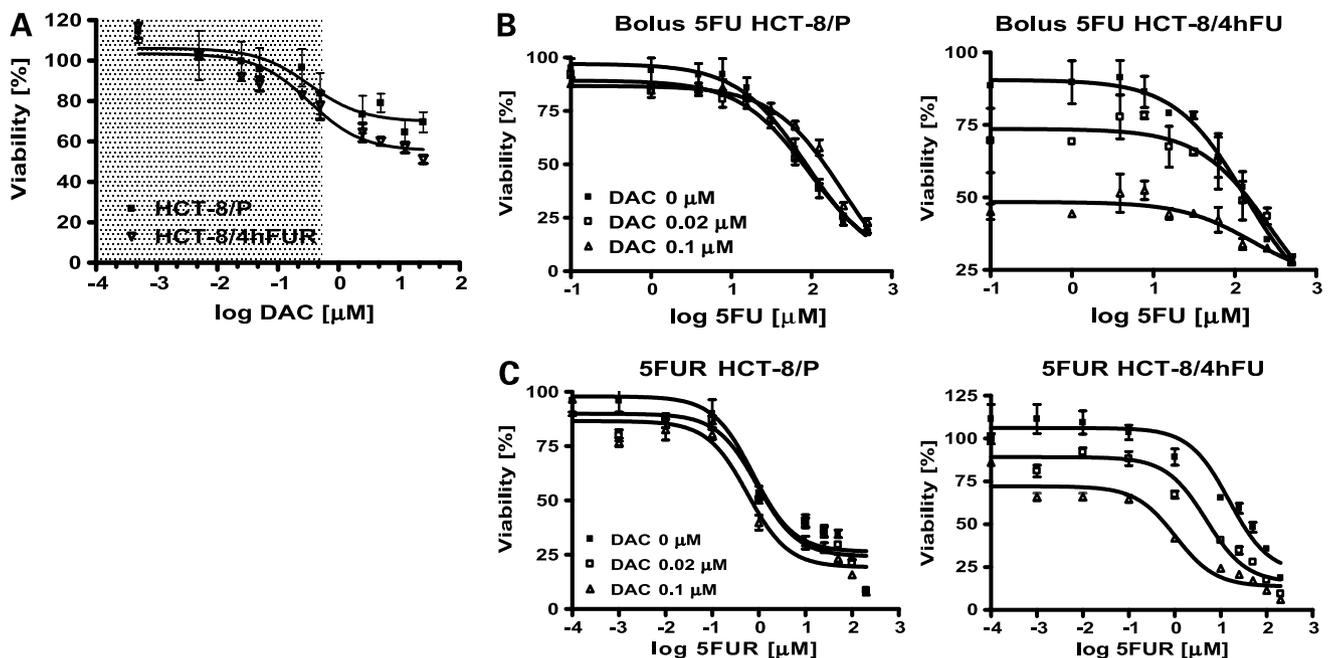


Figure 1. Pretreatment of HCT-8/4hFU cells with low doses of DAC restores its sensitivity to bolus 5-FU and 5-FUR treatments. **A**, HCT-8/P and HCT-8/4hFU cells were exposed to DAC for 24 h and cytotoxicity was assayed 4 d later. HCT-8/P (left) and HCT-8/4hFU (right) cells were exposed to 0.02 or 0.1 μmol/L DAC for 24 h and then treated for 4 h with various concentrations of 5-FU (**B**) or 5-FUR (**C**).

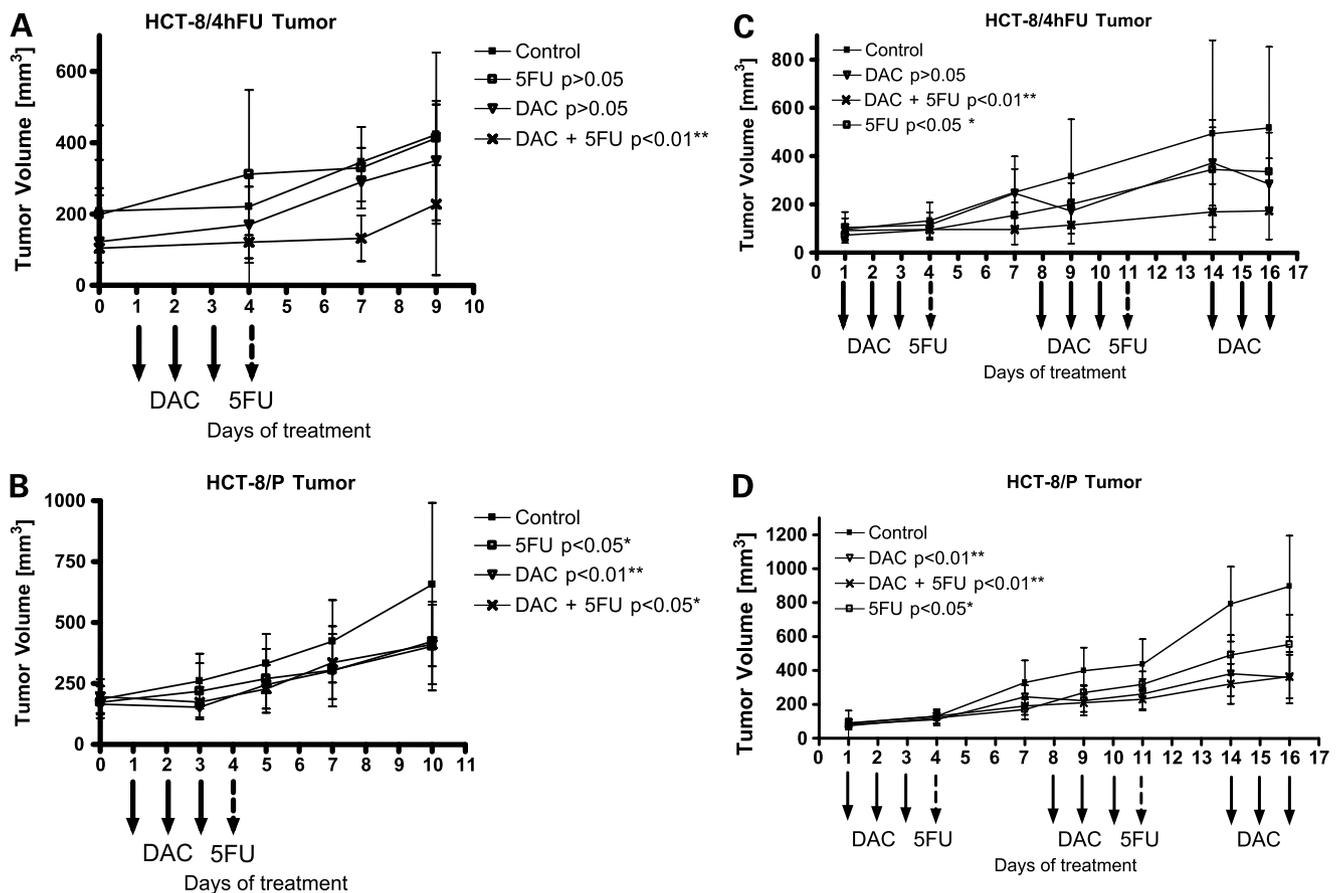


Figure 2. DAC enhances 5-FU activity in mouse colorectal cancer xenograft model. Mice bearing HCT-8/4hFU (**A** and **C**) or HCT-8/P (**B** and **D**) tumors were treated with DAC alone (3×0.5 mg/kg/course; empty triangle) or DAC followed by bolus 5-FU (1×50 mg/kg/course; cross), bolus 5-FU alone (1×50 mg/kg/course; open square), or saline (closed square) as a control. Animals received either one (8-10 animals per group; **A** and **B**) or two (5-8 animals per group; **C** and **D**) courses of treatment. Average \pm SD tumor volume for all of the animals. Two independent experiments are presented. Repeated-measures ANOVA test was used to assess statistical significance (P values for each group are given next to the figure legend).

in 96-well plates (Corning) the day before addition of the drug. Dilutions of 5-FU and 5-FUR were prepared fresh in RPMI 1640 before each use. Manufacturer's protocol was followed for preparation and use of MTS reagent.

Western Blotting

Cells were trypsinized, washed twice with PBS, and kept frozen in -80°C until used. Cell pellets or powdered tumors (see below) were thawed and resuspended in 0.5 mL radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 3 mmol/L sodium fluoride, and 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. Lysate (50 μg) was loaded onto 10% SDS-PAGE gel and after transfer to nitrocellulose probed with anti-UMPCK polyclonal antibody (1:1,000) for 2 h (gift of Dr. Y.C. Cheng, Yale University) and secondary anti-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 Reverse Transcription-PCR

Total RNA was extracted from 1×10^6 to 5×10^6 cells or powdered tumors 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 reverse transcription-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. $\Delta\Delta\text{Ct}$ method was used to calculate relative fold change in mRNA level (30). The sequence of UMPK forward primer is 5'-AAGAAGG-AAAGATTGTACCAGTTGAGA-3', reverse primer 5'-GGAA-ACCCATCAATCAAGAATTTATT-3', and probe 5-FAM-AGAGGGAAATGGATCAGACAATGGCTGC-TAMRA-3'.

Cloning of the UMPK Promoter Region

The sequence of 1,000 and 2,000 bp upstream of the UMPK translation initiation site was obtained from Ensemble Human Genome Data Resources at The Wellcome

Trust Sanger Institute (Fig. 4A). Primers were designed to amplify these regions from HCT-8/P and HCT-8/4hFU cells: *KpnI*MluIUMPK/P2000FP-5'-TTTATTGGTAC-CACGCGTACAAAGGCAAGTAGGTGAGG-3', *KpnI*MluIUMPK/P1000FP-5'-GCCGATTGGTAC-CACGCGTCTTTCCATAATCAAAAAGTCC-3', and *HindIII*XhoIUMPK/PRP-5'-AATAAAAAGCTTCTCGA-GACACCGCGCTCGGCCGGA-3'. Products were cloned into pGL-3 basic and enhancer vectors (Promega) that contain firefly luciferase gene as a reporter. Positive clones were confirmed by restriction digestion and sequencing. HCT-8/P and HCT-8/4hFU cells were transfected with the experimental constructs or an empty vector for background control (pGL-3 basic, pGL-3 enhancer). Cells were cotransfected with pRL-TK vector (*Renilla* luciferase) (Promega) in a 1:10 ratio as a control for transfection efficiency. The pGL-3 vector containing SV40 promoter was used as a positive control. Dual Luciferase Assay (Promega) was done 48 h after transfection and bioluminescence was recorded using a luminometer, TD-20/20 (Turner Design, DL Ready).

Determination of UMPK mRNA Decay

UMPK mRNA half-life was determined using a method based on real-time reverse transcription-PCR analysis (31). Briefly, transcription was inhibited using actinomycin D (5 μ g/mL) or 5,6-dichlorobenzimidazole ribose (25 μ mol/L). Total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration was determined using RiboGreen fluorescent dye (Molecular Probes, Invitrogen). Quality and integrity of total RNA were assessed on 1% formaldehyde-agarose gel. Total RNA (50 ng) was amplified in one-step reverse transcription-PCR (Applied Biosystems) using UMPK-specific primers and probe (as described above). Serial dilutions of plasmid containing UMPK cDNA (gift of Dr. Y.C. Cheng) were used to prepare a standard curve.

Colorectal Cancer Xenograft Model

HCT-8/P or HCT-8/4hFU cells (2.5×10^6) were injected s.c. into nude mice (Taconic). When tumors were palpable, animals were randomized into four groups (5-10 per group) and treated with DAC (Tuesday, Wednesday, and

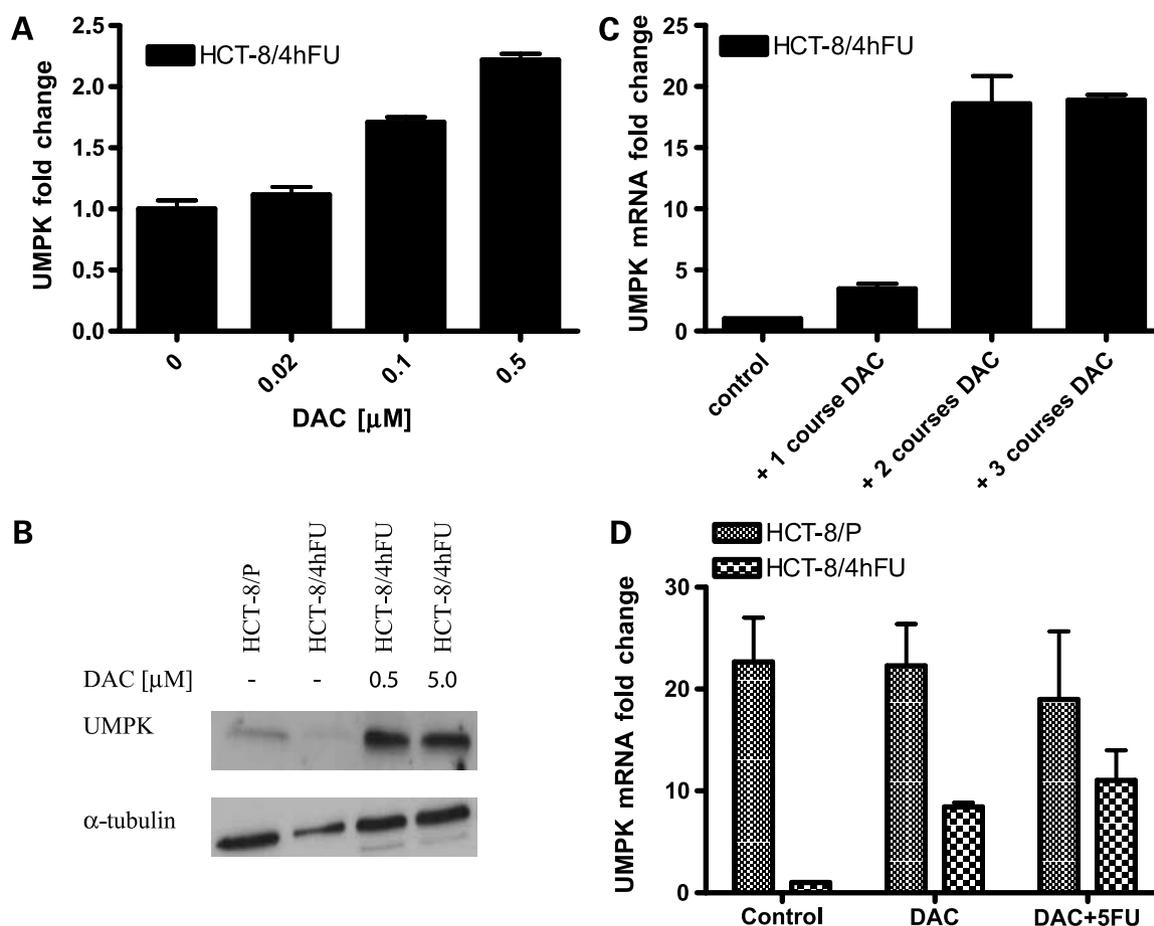


Figure 3. DAC treatment results in elevated UMPK levels. UMPK mRNA (A) and protein (B) levels were assayed in HCT-8/4hFU cells following 24 h exposure to DAC. Mice bearing HCT-8/4hFU tumors were given one, two, or three courses of DAC alone treatment (3×0.5 mg/kg/course). Twenty-four hours following DAC injection, tumors were harvested and UMPK mRNA level was compared with tumors harvested from untreated animals (C). Alternatively, the average induction of UMPK in all of the animals from the experimental groups, for which tumor volume is presented in Fig. 2C and D, was quantitated and expressed as fold change relative to the UMPK mRNA level in untreated HCT-8/4hFU tumors (D). Mean \pm SD.

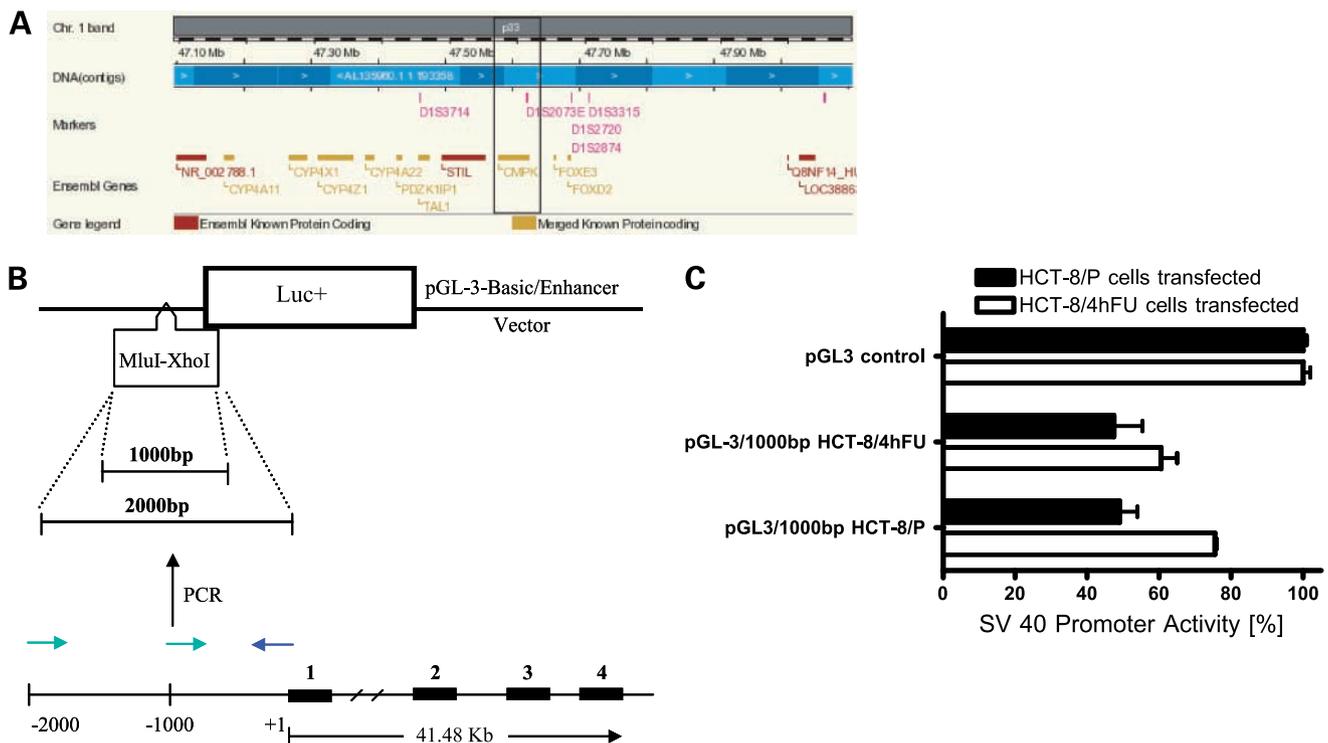


Figure 4. Cloning and expression of UMPK promoter region. **A**, location of UMPK gene denoted here as CMPK on chromosome 1. **B**, strategy used to clone UMPK promoter into pGL-3 vectors. Two forward primers and one reverse primer were designed to amplify 1,000 and 2,000 bp upstream of the first translation start site in exon 1 of UMPK gene. **C**, UMPK promoter activity compared with SV40 promoter (pGL-3 control vector). HCT-8/P or HCT-8/4hFU cells were transfected with constructs containing 1,000 bp regions amplified from HCT-8/P or HCT-8/4hFU genomic DNA and cloned into the pGL-3 enhancer vector.

Thursday; 0.5 mg/kg i.p.), 5-FU (Friday; 50 mg/kg i.p.), or DAC followed by 5-FU or PBS as a control. Measurements of tumor size and animal weight were done every 3 to 4 days. The schedule for DAC treatment that we used in this study has been published previously and was found to substantially increase UMPK gene expression in HCT-8/4hFU tumors (32). Two independent experiments were done and animals received either one or two weekly courses of treatment followed by an additional week of observation. To assay UMPK induction in tumors, animals from DAC alone and DAC and 5-FU groups received additional courses of DAC treatment toward the end of the experiment. Twenty-four hours after administration of the third dose of DAC, animals were anesthetized, and tumors were harvested, washed in PBS, flash frozen in liquid nitrogen, ground to a powder, and kept in liquid nitrogen until assayed. All experiments were done according to a protocol approved by The Institutional Animal Care and Use Committee at Robert Wood Johnson Medical School.

Pyro Q-CpG Analysis of Methylation

A search for CpG islands within UMPK promoter was done using Web-based software EBI Tools/CpG Plot (33). By default, this program defines a CpG island as a region where the calculated percentage composition is >50% and

the calculated observed/expected ratio is >0.6 and the conditions hold for a minimum of 200 bases. DNA was extracted from tumors or cells using standard phenol-chloroform-isoamyl alcohol extraction (Invitrogen). Sample DNA (2 μ g) or control DNA (1 μ g) was bisulfite modified using the EZ DNA Methylation Kit (Zymo Research). Post-bisulfite-modified DNA (1 of 10 μ L) was used for PCR. The region of the UMPK promoter that was amplified is shown in Fig. 5B. Bisulfite-treated fetal cell DNA and *in vitro* methylated human DNA (Chemicon, Millipore) were used in the assay development and PCR bias testing. The following reagents were mixed together in preparation for the pyrosequencing reaction: streptavidin beads (2 μ L), 2 \times binding buffer (40 μ L), PCR product (10 μ L), and water (28 μ L). The assay was done by EpigenDx using the PSQHS 96 System. Methylation of 42 CpG sites was analyzed using three sequencing primers to cover regions of interest. The average of r^2 for all the CpG sites was 0.96 showing that this assay was biased slightly toward unmethylated DNA.

The following primers and cycling condition were used: forward 5'-AATTATTTAAGGGTTGGAGGAAA-3' and reverse 5'-biotin-CTACAACAAAAAACTAAAACCCAA-3'. Cycling was at 95°C for 15 min; 45 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 30 s); 72°C for 10 min; 4°C.

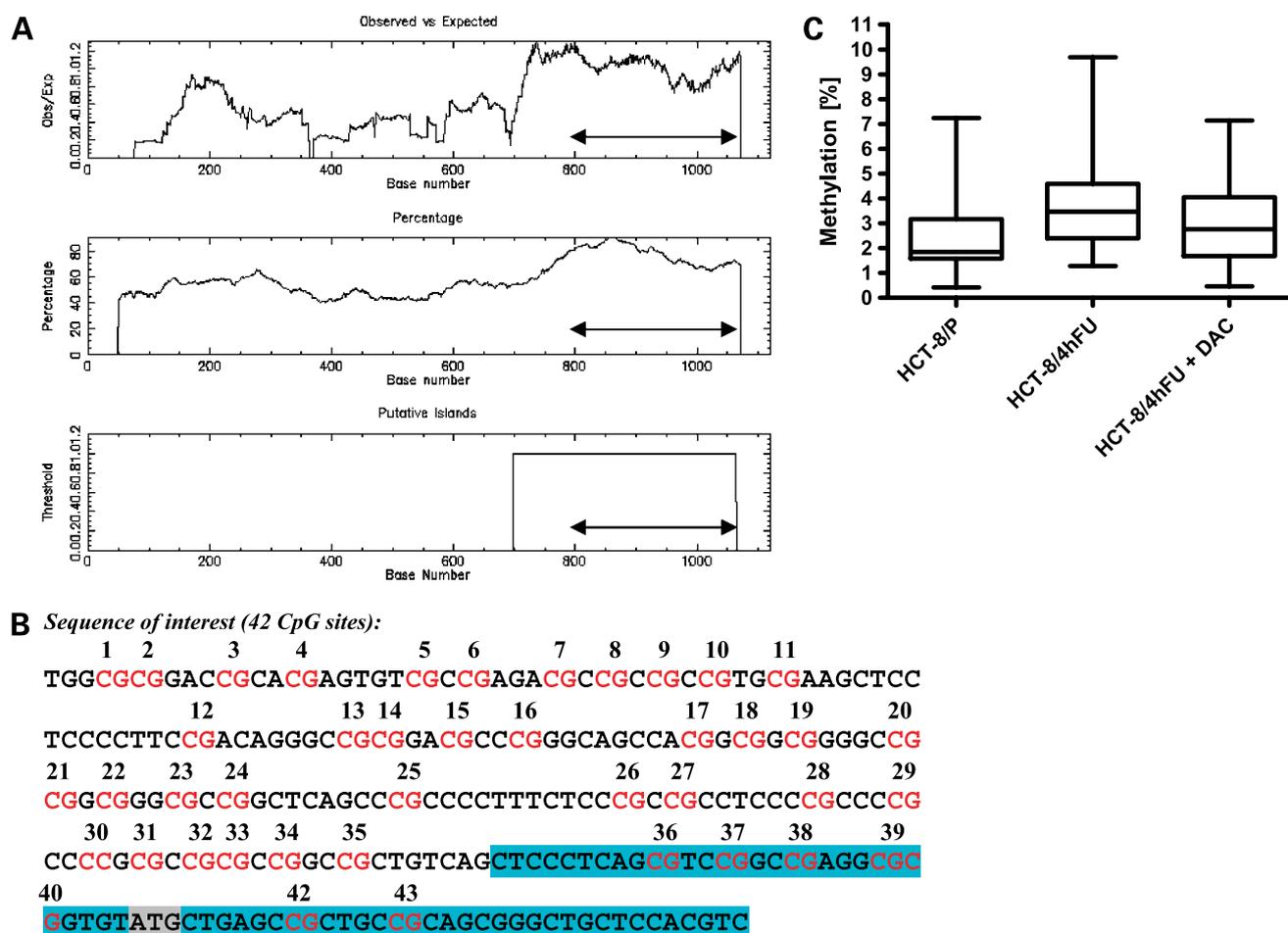


Figure 5. **A**, prediction of a CpG island located within the cloned 1,000 bp region containing UMPK promoter and the first exon. *Top*, observed/expected CpG ratio; *middle*, percentage of CpG dinucleotide; *bottom*, predicted CpG island. *Black arrow*, region of CpG island that was chosen for DNA methylation analysis. **B**, sequence of UMPK promoter region in which DNA methylation was analyzed. Methylation of total 42 CpG sites was analyzed. *Red*, CpG sites, with the representative numbers shown above each site; *blue*, beginning of UMPK transcript. **C**, average percentage of methylation across all of the 42 CpG sites analyzed in HCT-8/P and HCT-8/4hFU as well as HCT-8/4hFU cells treated with 0.5 $\mu\text{mol/L}$ DAC for 24 h. Median \pm minimum and maximum values.

Sequencing primer 1: 5'-TATTAAGGGTTGGAGGAAAGT-3'. Sequencing primer 2: 5'-GGAYGTTYGGGTAGTTA-3'. Sequencing primer 3: 5'-GTTYGTTYGYGTYGYGTYGG-TYGTGTTA-3'.

Statistical Analysis

GraphPad Prism 4 software was used for statistical analysis and statistical significance was assayed using the Student's *t* test or repeated-measures ANOVA in case of the analysis of delay in tumor progression.

Results

Subcytotoxic Doses of DAC Modulates Response to Bolus 5-FU and 5-FUR *In vitro*

Low doses of DAC are known to reactivate expression of genes silenced due to hypermethylation, whereas higher doses are cytotoxic (34). Because of the dual mechanism of

DAC action, we first explored its cytotoxicity profile toward HCT-8/P and HCT-8/4hFU cells (Fig. 1A). For further experiments, doses between 0.0005 and 0.5 $\mu\text{mol/L}$ were chosen that cause <20% of cell kill. We showed previously that HCT-8/4hFU cells are resistant to bolus 5-FU but sensitive to continuous 5-FU exposure and are cross-resistant to 5-FUR.³ 5-FUR and bolus 5-FU are predominantly incorporated into RNA and interfere with pre-rRNA processing (35). HCT-8/4hFU or HCT-8/P cells were pretreated with 0.02, 0.01, or 0.05 $\mu\text{mol/L}$ DAC for 24 h and then exposed to high doses of 5-FU or 5-FUR for 4 h (see

³R. Humeniuk, L.G. Menon, P.J. Mishra, et al. Probing the role of uridine monophosphate kinase in fluoropyrimidine resistance. *Mol Cancer Ther*, in press.

Materials and Methods). Cytotoxicity assays revealed that DAC pretreatment increases sensitivity of HCT-8/4hFU but not HCT-8/P cells to bolus 5-FU as well as 5-FUR treatment (Fig. 1B and C). The doubling time of HCT-8 parental and resistant cells is typically 16 h and our experiments showed that a 24 h DAC preincubation was sufficient to observe potentiation of 5-FU and 5-FUR cytotoxicity *in vitro*.

DAC Increases Potency of Bolus 5-FU in a Mouse Xenograft Model

Next, the effect of DAC on the response to bolus 5-FU was examined in a colorectal cancer xenograft model. HCT-8/4hFU and HCT-8/P cells were grown *s.c.* in nude mice. Animals were treated with low-dose DAC and bolus 5-FU as described in Materials and Methods. We used the repeated-measures ANOVA test to assess the statistical significance of our data and compare the responses over the

whole course of the treatment between the four treatment groups. To assure reliability of our data, two independent experiments were done because of a high variability in tumor growth rates that the HCT-8/4hFU cell line gives rise to. In the first experiment, one course of treatment was administered as shown in Fig. 2A and B. HCT-8/4hFU tumors were resistant to 5-FU alone or DAC alone treatments, but a significant delay in tumor growth was achieved when these drugs were given together ($P < 0.01$; Fig. 2A). HCT-8/P tumors responded to all of the treatments administered, and of interest, DAC alone treatment produced the most significant delay in tumor growth ($P < 0.01$; Fig. 2B). In the second experiment, two courses of treatment were administered followed by additional DAC injections (for assay of UMPK) before tumor harvest and the results are shown in Fig. 2C and D. Again, a significant growth delay of HCT-8/4hFU tumor was observed in

Table 1. Percentage of methylation of individual CpG sites analyzed within UMPK promoter and first exon

CpG	HCT-8/P	HCT-8/P + DAC	HCT-8/4hFU	HCT-8/4hFU + DAC
1	1.59 (0.10)	1.53 (0.12)	1.42 (0.21)	1.28 (0.11)
2	1.34 (0.33)	2.02 (0.01)	1.28 (0.12)	0.46 (0.46)
3	1.81 (0.09)	2.52 (0.22)	1.89 (0.11)	1.67 (0.00)
4	0.42 (0.42)	2.36 (0.71)	1.90 (0.15)	2.36 (0.17)
5	1.74 (0.18)	2.60 (0.08)	2.71 (0.11)	1.53 (0.35)
6	1.44 (0.18)	0.59 (0.59)	2.81 (0.10)	1.65 (0.20)
7	0.48 (0.48)	1.52 (0.38)	1.69 (0.07)	1.09 (1.09)
8	2.18 (0.11)	1.75 (0.27)	2.76 (0.19)	1.74 (0.56)
9	2.27 (0.10)	2.10 (0.59)	3.44 (0.49)	1.54 (0.27)
10	1.82 (0.19)	1.93 (0.17)	3.97 (0.11)	1.95 (0.12)
11	0.65 (0.65)	1.83 (0.47)	1.92 (0.17)	1.70 (0.58)
17	2.96 (0.10)	3.22 (0.08)	4.07 (0.33)	4.06 (0.20)
18	1.75 (1.75)	1.52 (1.52)	1.70 (1.70)	3.07 (1.04)
19	3.50 (1.42)	2.23 (2.23)	4.00 (1.72)	3.01 (3.01)
20	2.57 (2.57)	3.98 (0.56)	5.78 (0.19)	4.53 (1.14)
21	1.54 (1.54)	1.70 (1.70)	2.25 (2.25)	1.74 (1.74)
22	1.69 (1.69)	2.96 (0.98)	3.96 (0.77)	3.10 (1.14)
23	1.52 (1.52)	2.66 (0.50)	2.96 (0.63)	1.48 (1.48)
24	1.76 (1.76)	3.49 (0.69)	3.90 (0.38)	3.16 (0.32)
25	4.46 (0.81)	4.90 (0.73)	5.87 (0.60)	5.59 (1.54)
26	6.66 (0.27)	6.76 (0.73)	7.87 (0.40)	6.81 (0.86)
27	1.02 (1.02)	3.19 (0.28)	3.01 (0.16)	1.64 (1.64)
28	2.28 (2.28)	4.49 (0.87)	5.40 (0.32)	5.27 (0.17)
29	1.58 (1.58)	3.84 (0.22)	4.20 (0.49)	1.78 (1.78)
30	3.88 (0.00)	5.01 (0.00)	4.24 (0.00)	4.79 (0.00)
32	2.90 (0.00)	3.50 (0.00)	4.73 (0.00)	3.27 (0.00)
33	1.84 (0.00)	2.67 (0.00)	2.70 (0.00)	2.02 (0.00)
34	2.41 (0.00)	3.04 (0.00)	3.45 (0.00)	3.75 (0.00)
35	2.69 (0.00)	3.33 (0.00)	3.48 (0.00)	2.81 (0.00)
36	7.24 (0.00)	7.80 (0.00)	9.68 (0.00)	7.14 (0.00)
37	3.67 (0.00)	5.19 (0.00)	5.71 (0.00)	4.03 (0.00)
38	1.58 (0.00)	1.73 (0.00)	2.47 (0.00)	2.72 (0.00)
39	3.38 (0.00)	4.25 (0.00)	4.44 (0.00)	3.68 (0.00)
40	1.86 (0.00)	2.26 (0.00)	2.33 (0.00)	2.20 (0.00)
41	5.68 (0.00)	6.31 (0.00)	7.84 (0.00)	6.68 (0.00)
42	4.55 (0.00)	4.56 (0.00)	5.21 (0.00)	4.57 (0.00)

NOTE: Biological duplicates were analyzed and results were averaged. Mean (SD).

response to DAC followed by bolus 5-FU treatment compared with 5-FU alone ($P < 0.01$; Fig. 2C). No significant response of this tumor to the treatment with DAC alone and only a partial response to 5-FU alone was observed. HCT-8/P tumors, similar to the previous experiment, responded to all of the treatments administered, and unexpectedly, DAC alone or when followed by 5-FU produced the most significant effect ($P < 0.01$; Fig. 2D).

To compare HCT-8/P and HCT-8/4hFU tumor responses, both groups were treated on the same schedule. However, a shorter doubling time of the parental tumors could result in better DAC incorporation as well as 5-FU response of HCT-8/P tumors compared with HCT-8/4hFU tumors. Indeed, at comparable doses and schedules, we observed greater toxicity of treatment with DAC followed by 5-FU in HCT-8/P tumors compared with HCT-8/4hFU tumors (Supplementary Fig. S1).⁴ It is possible that even greater growth delay of HCT-8/4hFU tumors could be reached if the doses of both drugs or the schedule of administration was adjusted to reflect those differences in doubling time.

Effect of DAC Treatment on UMPK Expression *In vitro* and *In vivo*

We further explored the effect of DAC treatment on UMPK levels in the HCT-8/4hFU cells *in vitro* as well as in mouse xenografts bearing this tumor. A dose-dependent increase in UMPK mRNA as well as UMPK protein levels was observed in HCT-8/4hFU cells treated with low-dose DAC for 24 h (Fig. 3A and B). Note that the level of protein shown on Fig. 3B is a representative experiment and may not be directly correlated with the mRNA level shown in Fig. 3A. No increase in UMPK protein level (data not shown) and only a slight decrease in UMPK mRNA expression were observed in HCT-8/P cells (Supplementary Fig. S2).⁴ Analysis of mRNA isolated from HCT-8/4hFU tumors harvested at different time points following DAC administration showed a 4-fold increase in UMPK mRNA after one course of DAC treatment and up to a 20-fold increase after 2 and 3 weeks of treatment compared with untreated tumors (Fig. 3C). The average increase of UMPK in the group of animals bearing HCT-8/4hFU tumors was 8.5-fold in the DAC alone group and 11-fold in the DAC + 5-FU group (Fig. 3D). No further increase of UMPK after DAC treatment was observed in the HCT-8/P group. Interestingly, HCT-8/P tumors had, on average, 20-fold more UMPK mRNA than HCT-8/4hFU compared with only a 2-fold difference observed in the cultured cells.

Cloning of the UMPK Promoter Region

To study the mechanism of UMPK down-regulation in the HCT-8/4hFU cells and its reactivation mediated by

DAC treatment, we cloned the putative UMPK promoter. Regions (1,000 and 2,000 bp upstream) of the known UMPK translation start site were amplified from genomic DNA extracted from both HCT-8/P and HCT-8/4hFU cells and cloned into pGL-3 vectors (Fig. 4A and B). Sequencing of the cloned regions did not reveal any significant changes besides three single nucleotide polymorphisms of unknown function (data not shown). Expression of pGL-3 constructs in HCT-8/P cells showed that activities of the putative promoters are ~50% of the SV40 promoter activity and are the same for regions amplified from HCT-8/P as well as HCT-8/4hFU cell lines (Fig. 4C). When expressed in HCT-8/4hFU cells, the activity of the construct containing region amplified from HCT-8/P cells was only slightly higher and was not statistically different. Moreover, there was no significant difference between constructs containing 1,000 or 2,000 bp cloned into pGL-3 basic or enhancer vectors (data not shown). Based on these studies, we conclude that no functional changes between promoter regions exist between the sensitive and resistant cell lines.

Analysis of UMPK Promoter Methylation

Scanning of cloned 1,000 bp UMPK promoter region identified one CpG island surrounding the putative transcription start site. That location as well as observed/expected ratio and percentage of CpG dinucleotide is shown in Fig. 5A. A 240 bp region within the identified CpG island was chosen for further analysis of DNA methylation. Sequence and genomic context of this region is shown in Fig. 5B. A total of 42 CpG sites were analyzed within this sequence and 36 gave reliable results (verified by mixing studies). Enrichment in UMPK promoter DNA methylation was observed in HCT-8/4hFU cells compared with HCT-8/P cells. Moreover, treatment of HCT-8/4hFU cells with DAC resulted in the reversal of DNA methylation. Median percentage DNA methylation across 36 CpG sites that were analyzed in HCT-8/P, HCT-8/4hFU, and HCT-8/4hFU cells treated with 0.5 $\mu\text{mol/L}$ DAC for 24 h is shown in Fig. 5C. Mean percentage of methylation was 1.85, 3.47, and 2.74 for HCT-8/P, HCT-8/4hFU, and HCT-8/4hFU +DAC, respectively. Percentage of methylation at individual sites for both cell lines before and after treatment with DAC is shown in Table 1. Statistical analysis using the paired *t* test showed that observed changes in DNA methylation are statistically significant ($P < 0.0001$) compared with the percentage of methylation observed in untreated HCT-8/P cell line. Thus, a decrease in the UMPK mRNA level in the HCT-8/4hFU cells may be due to an increase in DNA methylation of the UMPK promoter that interferes with binding of transcription factors. However, an indirect effect of DAC on UMPK expression is also possible and may contribute to the observed increase in UMPK mRNA and protein levels.

UMPK mRNA Half-life Studies

To exclude the possibility that the observed decrease in UMPK mRNA and protein levels in HCT-8/4hFU cells is due to decreased message stability, half-life studies were carried out. Two different transcription inhibitors were

⁴Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

used (see Materials and Methods) and both showed that relative UMPK half-life is even longer in HCT-8/4hFU cells (34 h) than in HCT-8/P cells (20 h) excluding this possibility (Supplementary Fig. S3).⁴

Discussion

5-FU has been used to treat patients with colorectal, breast, and gastric cancers for >50 years. Initially, it was used as a single agent and more recently in combination with oxaliplatin, irinotecan, or bevacizumab (36, 37). However, acquired or intrinsic resistance still remains a major limitation to the clinical use of 5-FU and remains an area of active research. Here, we report that low-dose DAC reverses bolus 5-FU resistance *in vitro* and in a colorectal cancer xenograft model and that the restoration of the sensitivity is associated with increased expression of the 5-FU anabolizing enzyme, UMPK. The metabolism and mechanism of 5-FU action differ depending on the dose and schedule of administration (38). Bolus treatment results in drug anabolism mainly to fluoro-UTP, which is incorporated into RNA, interfering with normal pre-rRNA processing (39, 40). We observed that DAC also sensitizes HCT-8/4hFU cells to treatment with 5-FUR, a fluoropyrimidine that is known to be mainly incorporated into RNA. In the accompanying article, we show that short-term exposure of HCT-8 cells to repeated high doses of 5-FU resulted in acquisition of a low-grade resistance characterized by decreased expression of UMPK. Moreover, we have observed that colorectal cancer hepatic metastasis of patients previously exposed to 5-FU showed a high frequency of low or undetectable expression of UMPK.³

The increase in UMPK level following DAC treatment was dose and time dependent and was observed only in case of the bolus 5-FU-resistant tumors but not in the parental tumors that had already elevated level of this enzyme. Hence, the significant effect of DAC on 5-FU exposure observed in HCT-8/P tumors is UMPK independent and may be due to the significant effect that DAC alone treatment had on parental tumor. The preferential response of HCT-8/P tumor to DAC might be explained by better incorporation of the drug into DNA due to shorter doubling time of this tumor compared with HCT-8/4hFU tumor. The effect of DAC alone on tumor growth delay was transient, especially in case of HCT-8/4hFU tumor, and both tumors grew considerably in the intervals between DAC treatments. Administration of 5-FU following DAC treatment maintained the tumor response until the next treatment period or even after the treatment was discontinued.

Sequencing and expression of the UMPK promoter region revealed no functional changes between the two cell lines. Evaluation of 42 CpG sites within the island surrounding the putative transcription start site showed modest enrichment in DNA methylation across this region in HCT-8/4hFU cells that reverted following DAC treatment. However, due to a low percentage of methylation

observed, it is not clear whether DNA methylation is the only or even a factor responsible for lower expression of UMPK. Alternative transcriptional silencing pathways may exist. In the present studies, we examined the effect of DAC on UMPK promoter DNA methylation in the HCT-8 cell line only and the results may be unique to this particular cell line and may not be representative of all colorectal carcinoma cell lines. It is also possible that because of differences in folates present in cell culture conditions and differences in serum folates between mice and humans, the reversal of 5-FU resistance noted may not be predictive for human tumors *in vivo*. Unfortunately, because of the limited amount of tissue available to us, the examination of UMPK promoter methylation in fresh human tumor samples described in the accompanying article was not feasible.

DAC has been shown previously to reactivate genes even in the absence of promoter DNA methylation (41–43). Further characterization of the UMPK promoter and its gene regulation may provide more insight into the mechanism of this gene reactivation following DAC treatment. It has been reported that the IFN signaling pathway is highly inducible by DAC treatment and may be mediated by the induction of signal transducers and activators of transcription 1 to 3 (44, 45). Interestingly, IFN- γ has been shown to up-regulate the activities of the 5-FU anabolic enzyme thymidine phosphorylase and uridine phosphorylase resulting in enhanced 5-FU activation (36). Future studies will address the possibility of UMPK regulation through IFN signaling as well as identification of additional candidate genes or microRNAs that may be involved in conferring 5-FU sensitivity in colorectal cancer cells following DAC treatment.

Our results support the role of UMPK in acquired resistance to 5-FU and that resistance to bolus 5-FU in colorectal cancer can be overcome by combination therapy with low-dose DAC. Clinical studies will address the utility of treatment with DAC together with 5-FU in the reversal of 5-FU-induced resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Laird PW, Jackson-Grusby L, Fazeli A, et al. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell* 1995;81:197–205.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415–28.
- Chuang JC, Jones PA. Epigenetics and microRNAs. *Pediatr Res* 2007; 61:24–9R.
- Feinberg AP. The epigenetics of cancer etiology. *Semin Cancer Biol* 2004;14:427–32.
- Jen J, Powell SM, Papadopoulos N, et al. Molecular determinants of dysplasia in colorectal lesions. *Cancer Res* 1994;54:5523–6.
- Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–54.
- Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;16:6–21.

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8. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 2006;6:107–16.
9. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999;96:8681–6.
10. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;38:787–93.
11. Lee S, Hwang KS, Lee HJ, Kim JS, Kang GH. Aberrant CpG island hypermethylation of multiple genes in colorectal neoplasia. *Lab Invest* 2004;84:884–93.
12. Deng G, Song GA, Pong E, Sleisenger M, Kim YS. Promoter methylation inhibits APC gene expression by causing changes in chromatin conformation and interfering with the binding of transcription factor CCAAT-binding factor. *Cancer Res* 2004;64:2692–8.
13. Aguilera O, Fraga MF, Ballestar E, et al. Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. *Oncogene* 2006;25:4116–21.
14. Arnold CN, Goel A, Boland CR. Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. *Int J Cancer* 2003;106:66–73.
15. Deng G, Chen A, Pong E, Kim YS. Methylation in hMLH1 promoter interferes with its binding to transcription factor CBF and inhibits gene expression. *Oncogene* 2001;20:7120–7.
16. Liang JT, Chang KJ, Chen JC, et al. Hypermethylation of the p16 gene in sporadic T₃N₀M₀ stage colorectal cancers: association with DNA replication error and shorter survival. *Oncology* 1999;57:149–56.
17. Jacinto FV, Ballestar E, Ropero S, Esteller M. Discovery of epigenetically silenced genes by methylated DNA immunoprecipitation in colon cancer cells. *Cancer Res* 2007;67:11481–6.
18. Glasspool RM, Teodoridis JM, Brown R. Epigenetics as a mechanism driving polygenic clinical drug resistance. *Br J Cancer* 2006;94:1087–92.
19. Wei SH, Brown R, Huang TH. Aberrant DNA methylation in ovarian cancer: is there an epigenetic predisposition to drug response? *Ann N Y Acad Sci* 2003;983:243–50.
20. Soengas MS, Capodieci P, Polsky D, et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 2001;409:207–11.
21. Fulda S, Kufer MU, Meyer E, van Valen F, Dockhorn-Dworniczak B, Debatin KM. Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene* 2001;20:5865–77.
22. Nakayama M, Wada M, Harada T, et al. Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. *Blood* 1998;92:4296–307.
23. Gifford G, Paul J, Vasey PA, Kaye SB, Brown R. The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. *Clin Cancer Res* 2004;10:4420–6.
24. Kantarjian H, Issa JP, Rosenfeld CS, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer* 2006;106:1794–803.
25. Schrupp DS, Fischette MR, Nguyen DM, et al. Phase I study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. *Clin Cancer Res* 2006;12:5777–85.
26. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. *Cancer Res* 2000;60:6039–44.
27. Appleton K, Mackay HJ, Judson I, et al. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *J Clin Oncol* 2007;25:4603–9.
28. Aschele C, Sobrero A, Faderan MA, Bertino JR. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res* 1992;52:1855–64.
29. Pizzorno G, Handschumacher RE. Effect of clinically modeled regimens on the growth response and development of resistance in human colon carcinoma cell lines. *Biochem Pharmacol* 1995;49:559–65.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) method. *Methods* 2001;25:402–8.
31. Leclerc GJ, Leclerc GM, Barredo JC. Real-time RT-PCR analysis of mRNA decay: half-life of β-actin mRNA in human leukemia CCRF-CEM and Nalm-6 cell lines. *Cancer Cell Int* 2002;2:1.
32. Belinski SA, Klinge DM, Stidley CA, et al. Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. *Cancer Res* 2003;63:7089–93.
33. Rice P, Longden I, Bleasby A. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 2000;16:276–7.
34. Kantarjian HM, Issa JP. Decitabine dosing schedules. *Semin Hematol* 2005;42:S17–22.
35. Wilkinson DS, Tlsty TD, Hanas RJ. The inhibition of ribosomal RNA synthesis and maturation in Novikoff hepatoma cells by 5-fluorouridine. *Cancer Res* 1975;35:3014–20.
36. Longley DB, Harkin DP, Johnston PG. 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003;3:330–8.
37. Pasetto LM, Bortolami A, Falci C, Sinigaglia G, Monfardini S. Recent progress in target therapy in colorectal cancer. *Anticancer Res* 2006;26:3973–81.
38. Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokinet* 1989;16:215–37.
39. Sobrero AF, Aschele C, Bertino JR. Fluorouracil in colorectal cancer—a tale of two drugs: implications for biochemical modulation. *J Clin Oncol* 1997;15:368–81.
40. Fang F, Hoskins J, Butler JS. 5-Fluorouracil enhances exosome-dependent accumulation of polyadenylated rRNAs. *Mol Cell Biol* 2004;24:10766–76.
41. Zhu WG, Dai Z, Ding H, et al. Increased expression of unmethylated CDKN2D by 5-aza-2'-deoxycytidine in human lung cancer cells. *Oncogene* 2001;20:7787–96.
42. Hummel M, Yan S, Li Z, Varghese TK, Abecassis M. Transcriptional reactivation of murine cytomegalovirus in gene expression by 5-aza-2'-deoxycytidine and trichostatin A in latently infected cells despite lack of methylation of the major immediate-early promoter. *J Gen Virol* 2007;88:1097–102.
43. Ibanez de Caceres I, Dulaimi E, Hoffman AM, Al-Saleem T, Uzzo RG, Cairns P. Identification of novel target genes by an epigenetic reactivation screen of renal cancer. *Cancer Res* 2006;66:5021–8.
44. Karpf AR, Peterson PW, Rawlins JT, et al. Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. *Proc Natl Acad Sci U S A* 1999;96:14007–12.
45. Mikovits JA, Young HA, Vertino P, et al. Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in de novo methylation of the γ interferon (IFN-γ) promoter and subsequent downregulation of IFN-γ production. *Mol Cell Biol* 1998;18:5166–77.

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