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Modulation of human UMP/CMP kinase affects activation and cellular sensitivity of deoxycytidine analogs

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ABSTRACT

Deoxycytidine analogs are an important class of clinically active antiviral and anticancer agents. The stepwise phosphorylation of these analogs to triphosphate metabolites is crucial for biological action. Human UMP/CMP kinase (UMP/CMPK; cytidylate kinase; EC 2.7.4.14) is thought to be responsible for phosphorylation of UMP, CMP, and dCMP and may also play an important role in the activation of pyrimidine analogs. However, no evidence has verified this notion in intact cells. In this study we explored the functional roles of UMP/CMPK in natural pyrimidine synthesis and metabolism of deoxycytidine analogs, as well as 5-FU in HeLa S3 and HCT8 cells. The amounts of UMP/CMPK protein in different cell lines correlated with UMP, CMP, and dCMP kinase activities and amounts of UMP/CMPK RNA. Modulation of UMP/CMPK by overexpression or down-regulation had no impact on natural pyrimidine nucleotides and cell growth. However, down-regulating UMP/CMPK expression by siRNA led to a decrease in the formation of the triphosphate metabolites, resulting in cellular resistance to these analogs. More diphosphate and triphosphate metabolites of deoxycytidine analogs were detected and cellular sensitivity to these agents was increased in the UMP/CMPK-overexpressing cells. This study indicates that the second step enzyme (UMP/CMPK) is responsible for the phosphorylation of pyrimidine analogs and also has an impact on cellular sensitivity to these analogs in those cell lines.

1. Introduction

UMP/CMP kinase (UMP/CMPK; EC 2.7.4.14) catalyzes the phosphorylation of UMP, CMP, and dCMP to the respective diphosphates using ATP as a phosphate donor. Salvage and *de novo* syntheses of pyrimidines converge at this enzyme to produce diphosphates from the monophosphate forms [1]. UMP/CMPK may thus be crucial for cellular nucleic acid synthesis. UMP/CMPK from different species is highly conserved, suggesting an important role of the kinase in all of these organisms. A conditional lethal mutant isolated from *Saccharomyces cerevisiae* has been identified from mutated UMP/CMPK, indicating that this enzyme might be essential in the survival of this organism and possibly in mammalian cells [2].

UMP/CMPK may also play an important role in the activation of deoxycytidine analogs, many of which are important anticancer and antiviral agents [3–5]. For example, 5-fluorouracil (5-FU) is widely used in the treatment of cancers, including colorectal and breast cancers [6,7]. 1-β-D-arabinofuranosylcytosine (araC) is commonly used to treat hematologic malignancies [8]. 2′,2′-difluorodeoxycytidine (dFdC; gemcitabine) has been shown to be active against pancreatic cancer and several other solid tumors [9,10]. β-L-dioxaolanecytidine (L-OddC), a deoxycytidine analog with an unnatural L-configuration, is currently under active clinical investigation because of promising antitumor effects seen in preclinical models and early clinical studies [11–14]. The analogs need to be phosphorylated stepwise to the triphosphate forms to exert therapeutic effects. Studies have suggested that UMP/CMPK is responsible for the phosphorylation of these analogs from monophosphates to the diphosphate metabolites [15,16].

Abbreviations: 5-FU, 5-fluorouracil; 5-FUR, 5-fluorouridine; araC, 1-β-D-arabinofuranosylcytosine; dC, β-D-2′-deoxycytidine; Gem, 2′,2′-difluorodeoxycytidine, gemcitabine; MP, monophosphate; DP, diphosphate; TP, triphosphate; dCK, deoxycytidine kinase; CMPK, CMP kinase; dCMPK, dCMP kinase; UMPK, UMP kinase; NDPK, nucleoside diphosphate kinase; dNTP, deoxynucleoside triphosphate.

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Human UMP/CMPK has been cloned and characterized by several groups, including our group [15–19]. Kinetic studies of recombinant human UMP/CMPK have shown that CMP and UMP are much better substrates than dCMP [15–17,19]. The relative efficiency of phosphorylating dCMP by this enzyme is about 100-fold less efficient than that of CMP or UMP. This finding is consistent with previous studies using partially purified enzymes from human cancer cells [20–22]. The phosphorylation of various deoxycytidine analog monophosphates could be carried out by recombinant human UMP/CMPK *in vitro*, but only with efficiencies comparable to or less than the rate of phosphorylation of dCMP [15]. However, no evidence has yet demonstrated that UMP/CMPK is responsible for the phosphorylation of various deoxycytidine analog monophosphates and natural pyrimidine monophosphates in intact cells, as well as for the impact of UMP/CMPK on cell growth. Although only one UMP/CMPK gene has been cloned, the possibility of the presence of unidentified cellular dCMP kinases remains and should be explored in mammalian cells.

In this study, we examined the amounts of UMP/CMPK protein, which were correlated with UMP, CMP, and dCMP kinase activities and amounts of mRNA of UMP/CMPK in various human cell lines. We modulated UMP/CMPK expression in HeLa S3 and HCT8 cells to investigate its impact on natural pyrimidine nucleotides, cell growth, and the phosphorylation of clinically active anticancer pyrimidine analogs. We further elucidated that the level of expression of UMP/CMPK would affect the sensitivity to the anticancer pyrimidine analogs in those two cells.

2. Materials and methods

2.1. Western blotting

SDS-polyacrylamide gel electrophoresis was performed, as described by Laemmli [23]. The protein was then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a Miniprotein II transferring apparatus (Bio-Rad Laboratories). The membranes were blocked and probed in PBS-T buffer (1× PBS buffer and 0.2% Tween-20) containing 5% non-fat milk. The immunoreactive bands were visualized using enhanced chemiluminescence reagents (PerkinElmer Life Science Products, Foster City, CA, USA), and densitometry scanning was performed using the PhosphoImager (Amersham Biosciences, Piscataway, NJ, USA). Anti-dCK antibody was a kind gift of Dr. Staffan Eriksson (SLU, The Biomedical Center, Uppsala, Sweden). Rabbit anti-UMP/CMPK antiserum was produced by our laboratory [15]. Rabbit anti-nm-23 polyclonal antibody was purchased from Neomarkers Inc. (Fremont, CA, USA).

2.2. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA). Quantitative RT-PCR for UMP/CMPK was conducted using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) with the UMP/CMPK-specific primers 5′-TCTCATGAAGCCGCTGGTCGT-3′ and 5′-TCTGCCTTCC-CATCCATGGTCT-3′, according to the manufacturer's instructions on a Rotor-Gene RG 3000 system (Corbett Research, Sydney, Australia). To ensure that the correct product was amplified in the reaction, all samples were examined by 1.5% agarose gel electrophoresis. The transcript was quantified relative to the transcript glyceraldehyde-3-phosphate dehydrogenase. The relative gene expression of UMP/CMPK in various cell lines was calculated by the ΔCt method. The average Ct value of the GAPDH gene was subtracted from the average Ct value of UMP/CMPK ($\Delta Ct = Ct_{UMP/CMPK} - Ct_{GAPDH}$). The relative expression of each cell line was further normalized to the HeLa S3 cell line as the ΔCt value of each cell line subtracted from the ΔCt of the HeLa S3 cell line (ΔΔCt). The normalized ratios are calculated as $2^{-\Delta\Delta Ct}$. The primers used for glyceraldehyde-3-phosphate dehydrogenase were as follows: 5′-ATCACCATCTTCCAGGAGCGAGA-3′ and 5′-AGAGGCAGGGATGATGTTCTGGA-3′.

2.3. Growth inhibition assays

Cells were plated at 1×10^4 cells/well in 24-well plates or 4×10^3 cells/well in 48-well plates. Serially diluted drugs in triplicate were added to cells in logarithmic growth. After three-doubling times, cells were stained with 0.5% methylene blue in 50% ethanol, air dried, and the cell pellet was dissolved in 1% sarkosyl. The optical density at 600 nm was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell growth relative to control was calculated by dividing the OD_{600} values in drug-treated wells by those in control wells. Data were plotted as a percentage of cell growth versus drug concentration.

2.4. Enzyme activity assays

Cells in logarithmic growth were washed with PBS, and lysed on ice for 1 h using lysis buffer (25 mM Tris–HCl [pH 7.4], 25 mM NaCl, 5 mM NaF, 2 mM DTT, 0.5% Triton X-100, 0.5 mM EDTA, and protease inhibitors). After centrifuging at 13,500 rpm for 20 min, the extracts were isolated for protein analysis and enzyme activity assays. The enzyme assays were based on the DE-81 disc assay with minor modifications [24]. For dCK, we used dC and cladribine (CdA) (Sigma, St Louis, MO, USA) as substrates. The assay was performed in a reaction buffer consisting of 50 mM Tris–HCl [pH 7.5], 10 mM NaF, 2 mM DTT, 0.04 mM tetrahydrouridine, and 5 mM ATP/Mg^{2+}) supplemented with creatine phosphate and creatine kinase as ATP-regenerating systems. The substrates used were 50 μM [^{3}H]dC and [^{3}H]CdA (Moravek Biochemicals, Brea, CA, USA). The reaction was performed at 37 °C for 2 h with a 75 μl total mixture for each reaction containing 10–20 μl of each cell extract. A 50-μl aliquot from each reaction mixture was spotted on DE-81 discs (Whatman, Clifton, NJ, USA). The DE-81 discs were washed 3 times with 1 mM ammonium formate for 3 min, once with distilled water for 2 min, and then once with 95% ethanol for 2 min. To improve the detection of ^{3}H-labeled radioactive nucleotides, compounds were eluted from the discs by incubation with 0.1 N HCl plus 2 M NaCl for 20 min before being counted. For determining CMPK and dCMPK activities, the DE-81 disc assay was performed with minor modifications at the washing step [15]. The enzyme assays was performed in a buffer consisting of 50 mM Tris–HCl (pH 7.5), 10 mM NaF, 2 mM DTT, and 1 mM ATP/Mg^{2+}, supplemented with creatine phosphate and creatine kinase for regeneration of ATP in a total volume of 75 μl for each reaction. The discs were washed 3 times with washing solution (1 mM ammonium formate for UMP, CMP, and dCMP, plus 50 mM formic acid for dCMP and CMP, and 500 mM formic acid for UMP) for 3 min. The products were not washed off the discs to any significant degree (<5%) under the specified conditions. The discs were then washed once with 95% ethanol and subsequently dried. The discs were assayed for radioactivity as described above.

2.5. Measurement of NDPK activity in a coupled enzymatic assay

NDPK activities were examined in a kinetic coupled enzyme assay using the pyruvate kinase-lactate dehydrogenase method [25]. The assay measures ADP formation from ATP, with dTDP as the acceptor nucleotide. The enzyme rates were recorded for the first 2–5 min of the reaction, where the initial velocity (ΔA_{340}/min) was determined from the linear portion of the curve. The value of the control reaction was subtracted from the sample reaction. One

unit refers to the amount of enzyme converting 1 μmol of ADP/min at room temperature. NDPK activity is expressed as units/mg protein.

2.6. Cell culture, transfection, and cloning of stable transfectants

HeLa S3 (a human cervical carcinoma cell line) and HCT-8 (a human colon carcinoma cell line) cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 at 37 °C. Transfections using Lipofectamine 2000 (Invitrogen) were performed as outlined in the manufacturer's manual. The transfectants were selected with 1 mg/ml G418 (Invitrogen) from day 3, and continued for 3 weeks. The colonies were then selected and cultured in separate wells in 24-well plates. The activity of each clone was analyzed, and positive clones were further cloned by serial dilution.

2.7. siRNA transfection

2×10^5 HeLa S3 cells were plated into 6-well plates, 1 day before transfection (day 0). The next day (day 1), cationic lipid complexes were prepared by incubating 200 pmol/l siRNA with 6 μl of Oligofectamine (Invitrogen) in 200 μl of OPTI-MEM (Invitrogen) for 15 min. The complexes were added to the cells to a final volume of 1 ml. After incubation for 4 h, 0.5 ml of RPMI supplemented with 30% FBS was added to each well. The transfection was repeated on day 2. On day 3, cells from each well were replated in the absence of siRNA, based on the experimental requirements, as follows: (a) cells were plated into 6-cm dishes for carrying out the metabolism experiments; (b) cells were replated at 60% confluence into fresh 6-well plates for immunoblotting; (c) 2.5×10^4 cells were plated in cover slides (BD Biosciences, Bedford, MA, USA) for immunofluorescence; and (d) 200 cells were plated into 6-cm plates for clonogenic assays. The respective experiments were carried out 24 h after being replated. The siRNA sequences used were as follows: si128, sense 5′-CCCAGAUUCACAGUAUGGUdTdT-3′ and antisense 5′-ACCAUACUGUGAAUCUGGGdTdT-3′; si128inv, sense 5′-UGGUAUGACACUUAGACCCdTdT-3′ and antisense 5′-GGGUCUAAGUGUCAUACCAdTdT-3′; and si257, sense 5′-UAAAUUCUUGAUUGAUGGGdTdT-3′ and antisense 5′-CCCAUCAAUCAAGAAUUUAdTdT-3′.

2.8. Metabolism of nucleoside analogs

The cells were treated with the respective radioactive compounds at the indicated concentrations. The cells were harvested in cold phosphate-buffered saline containing 10 μM dipyridamole (Sigma) and extracted with 15% trichloroacetic acid for 10 min on ice. The supernatant containing the nucleoside and its phosphorylated forms was extracted with a 45/55 ratio of trioctylamine and 1,1,2-trichlorotrifluroethane. The trichloroacetic acid insoluble pellet representing the nucleotide incorporated into the DNA was washed twice and resolubilized in DMSO prior to evaluation on a Beckman LS5000TD scintillation counter (Beckmann Instruments, Fullerton, CA, USA). The nucleoside analog metabolites were analyzed by Agilent 1100 high pressure liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) connected to a radiomatic detector Flow Scintillation Analyzer 150TR (Packard, Meriden, CT, USA) using a Partisil SAX column (Whatman). All results are the means and standard deviations from at least three independent transfections.

2.9. Determination of NTP and dNTP pools

For NTP pools analysis, the cells were harvested and extracted with 60% methanol as described previously [26]. The extracts were centrifuged and the supernatants were collected. The supernatants were heated for 5 min at 95 °C, frozen at −80 °C, and kept at −80 °C after lyophilization. The nucleoside metabolites were dissolved in water and analyzed by HPLC. The amounts of dNTPs were determined by enzymatic assay using the Sequenase enzyme, as described [26]. Briefly, the reaction mixture for Sequenase (2.0) was prepared to contain 0.05 or 0.1 units of Sequenase, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 5 mM dithiothreitol. The reaction mixture also contained 0.25 μM template primer and 2.5 μM [3H]dATP (20 Ci/mmol) for dCTP and dTTP determination. Extract from approximately 2×10^5 cells in 5 μl was then added to 45 μl of the reaction mixture; the reaction was performed at 26 °C for 20 min. Forty microliters of the reaction mixture was spotted onto a Whatman DE-81 disc. The filters were extensively washed 3 times with 5% Na_2HPO_4 for 10 min, then rinsed with distilled water. The disc was air dried after rinsing with 95% ethanol. The radioactivity on the disc was extracted and counted as mentioned above.

2.10. Clonogenic assay

Two hundred cells were treated with the indicated agents for 4 h. Cells were washed twice with PBS, and fresh media was added. The colonies were counted 10 days later by staining the colonies with methylene blue. The results are the means and standard deviations from three-to-five independent transfections.

2.11. Statistical analysis

The results are expressed as the mean with SD. The statistical significance between groups was determined using the unpaired Student's *t*-test. p values <0.05 were considered to indicate statistical significance. The Pearson correlation coefficient and statistics are calculated by SPSS (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. UMP/CMPK protein levels correlate with kinase activities and the RNA levels in cells

UMP/CMPK protein levels and kinase activities were examined in various human cell lines. UMP/CMPK protein was expressed differentially in different cell lines, as HCT8 and WI-38 cell lines expressed less than the other cell lines (Fig. 1(A)). We also found normal cells express less UMP/CMPK protein than cancer cells (WI-38, HUVEC, and HEK293 cells; data not shown). When the kinase activities of the cell lines were analyzed, we found that UMP, CMP, and dCMP kinase activities correlated well with UMP/CMPK protein levels (Fig. 1(A)–(C)). The correlation coefficients of the UMP/CMPK protein level versus the UMP, CMP, and dCMP kinase activities were 0.948, 0.958, and 0.982, respectively; all three p values were <0.01. These results suggest that the UMP/CMPK protein is the major kinase detected for the phosphorylation of UMP, CMP, and dCMP in the cellular extract. To determine whether the difference in the expression of the UMP/CMPK protein is regulated at the transcription level, quantitative RT-PCR was performed to quantify the UMP/CMPK RNA levels in different cells. The RNA levels of UMP/CMPK correlate well with the protein level in various cell lines (Fig. 1). The correlation coefficient of the UMP/CMPK protein level versus the mRNA level was 0.779 ($p < 0.05$).

3.2. The impact of UMP/CMPK down-regulation on pyrimidine nucleotides and sensitivity to anticancer pyrimidine analogs

UMP/CMPK is suggested to be the enzyme that phosphorylates UMP, CMP, dCMP, and anticancer pyrimidine analogs in the cells; however, no direct evidence has been provided previously. To

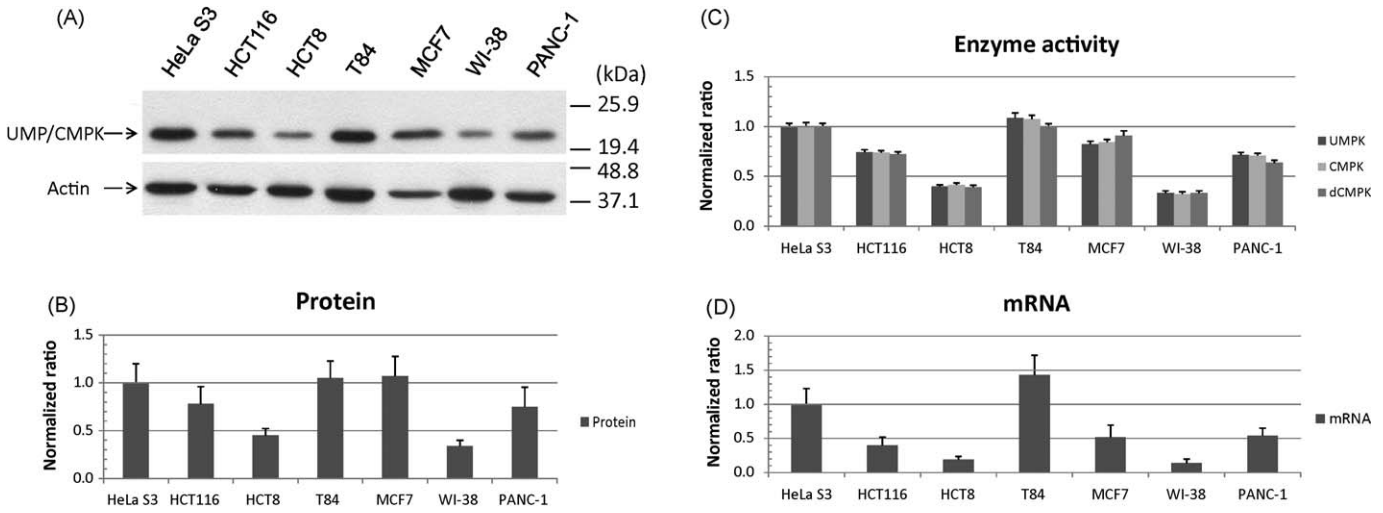

Fig. 1. UMP/CMPK protein, enzyme activities, and mRNA levels in various human cell lines. (A) Level of expression of UMP/CMPK protein. Cell lysates from different human cell lines were prepared and analyzed by Western blotting. UMP/CMPK and actin bands are indicated. The immunoblotting picture shown is a representative of three independent experiments. (B) Normalized UMP/CMPK/actin ratio. Band intensities of UMP/CMPK and actin of Western blotting were quantified by densitometry. Ratios of UMP/CMPK over actin of band intensities are calculated. The ratios are further normalized with that of HeLa S3 cells and presented as bar graphs. Each bar graph represents the mean from three independent experiments; SD is shown as a vertical bar. (C) UMPK, CMPK, and dCMPK enzyme activities in cell lysates. Cell lysates were prepared and kinase activities were assayed as described in Section 2. Activity values are normalized with that of HeLa S3 cells and the normalized ratios are presented as bar graphs. Each bar graph represents the mean from at least three independent experiments; SD is shown as a vertical bar. UMPK, CMPK, and dCMPK activities of HeLa S3 cells are 33.2 ± 1.1, 29.1 ± 1.2, and 23.4 ± 0.8 nmol/min/mg protein, respectively. (D) Level of expression of UMP/CMPK mRNA. Relative gene expression of UMP/CMPK was determined by quantitative real-time RT-PCR. The relative expression of each cell line normalized to that of HeLa-S3 cell is calculated as described in Section 2. The normalized ratios are shown as bar graphs. Each bar graph represents the mean from at least three independent experiments; SD is shown as a vertical bar.

explore the functional role of UMP/CMPK in cells, we designed three siRNAs to down-regulate UMP/CMPK protein in cells. Two of these siRNAs were effective in knocking-down UMP/CMPK, as si128 was shown to be the most effective siRNA (>80% down-regulation) and was thus chosen for further experiments (Fig. 2 and data not shown). si128 could down-regulate >80% of the level of expression of UMP/CMPK protein in several cell lines tested, including HeLa S3; the down-regulation could be maintained >3 days (data not shown). The transfection efficiency was approximately 90%, as examined by immuno-fluororescent microscopy (data not shown). The UMP, CMP, and dCMP kinase activities were shown to have decreased >70%, and correlated with the level of UMP/CMPK protein determined by Western blotting. The protein level and kinase activity of dCK were examined, and the results showed that the protein level and kinase activity of dCK were not affected by UMP/CMPK down-regulation (Fig. 2 and Table 1). The control and UMP/CMPK-down-regulated cell lysates showed no difference in total NDPK activity and protein levels of two major cytosolic NDPKs (NM23 H1 and H2). With respect to drug sensitivity, down-regulation of UMP/CMPK protein decreased cellular sensitivity to gemcitabine (resistant factor [RF] = 3.5; $p < 0.01$), cytarabine (RF = 2.8; $p < 0.01$), 5-FU (RF = 2.1; $p < 0.05$), and FUR (RF = 1.8; $p < 0.05$), as determined by clonogenic assays (Fig. 3 and Table 2). However, cellular sensitivity to non-related drug camptothecin (CPT) was not affected, suggesting the effect was specific. Since *de novo* and salvage syntheses of pyrimidine converge at UMP/CMPK, down-regulation of UMP/CMPK was expected to have an impact on pyrimidine triphosphate pools and DNA synthesis, and thus also on cell growth. Cellular nucleotides were extracted and analyzed by HPLC. NTPs amounts were almost the same in control and UMP/CMPK-down-regulated cells (UTP = 1.34 ± 0.51 *vs.* 1.22 ± 0.38 nmol/10^6 cells; CTP = 0.42 ± 0.16 *vs.* 0.41 ± 0.08 nmol/10^6 cells; ATP = 2.80 ± 0.74 *vs.* 2.69 ± 0.49 nmol/10^6 cells; and GTP = 0.73 ± 0.31 *vs.* 0.68 ± 0.03 nmol/10^6 cells). We further examined dCTP and TTP amounts by polymerase reaction assay in these cells, and found that their levels were almost the same in these cell lines (dCTP = 3.9 ± 0.5 *vs.* 3.8 ± 0.5 pmol/10^6 cells; and dTTP = 34.1 ± 1.5 *vs.* 33.8 ± 1.7 pmol/10^6 cells).

3.3. Down-regulation of UMP/CMPK decreases phosphorylation of anticancer deoxycytidine monophosphate in cells

Down-regulation of UMP/CMPK causes cells to be more resistant to the anticancer pyrimidine analogs examined; this might be due to less phosphorylation of pyrimidine monophosphates to diphosphates and triphosphates. Cells were incubated

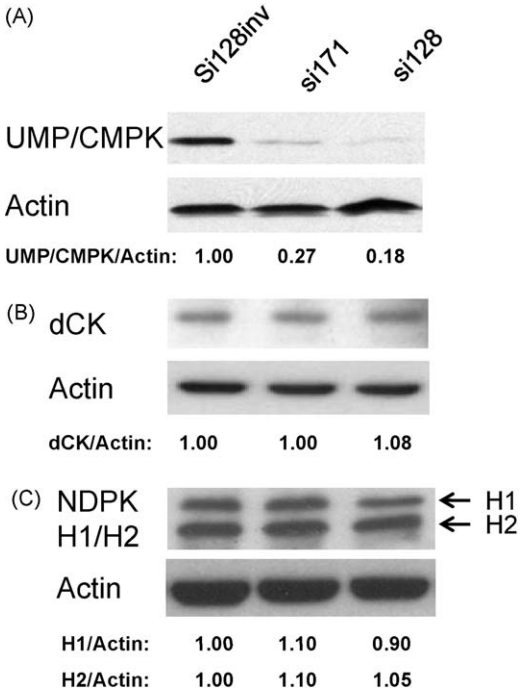

Fig. 2. Level of expression of UMP/CMPK (A), dCK (B), and NDPK H1/H2(C) protein in si128inv, si171, and si128 siRNA-transfected HeLa S3 cells, respectively. Cell lysates were prepared and analyzed by Western blotting as described. Normalized values of UMP/CMPK/actin ratio are presented. Immunoblotting pictures shown are representative of three independent experiments.

Table 1
dCK, UMPK, CMPK, dCMPK, and NDPK activities of si128inv (control), si171, and si128 siRNA-transfected HeLa S3 cells. Enzyme activity assays were performed as described. Values are presented as mean ± SD from three to five independent experiments.

Activity	si128inv	si171	si128
UMPK[a]	36.3 ± 2.1	13.6 ± 1.4	11.2 ± 0.7
CMPK[a]	30.5 ± 1.8	11.6 ± 1.2	9.5 ± 0.5
dCMPK[a]	23.1 ± 1.1	9.4 ± 0.8	7.2 ± 0.5
dCK[b]	68.5 ± 1.7	66.4 ± 1.8	69.8 ± 2.1
NDPK[c]	1.43 ± 0.33	1.40 ± 0.23	1.58 ± 0.26

[a] nmol/min/mg protein.
[b] pmol/min/mg protein; CdA as a substrate.
[c] unit/mg protein.

Table 2
The 50% inhibitory concentrations of gemcitabine, cytarabine, 5-FU, FUR, and CPT of si128inv (control) and si128 siRNA-transfected HeLa S3 cells. The data were derived from clonogenic assays. Values represent the means ± SD of five independent experiments.

Drug	Si128 Ave ± SD	Si128inv Ave ± SD	RF
Gem (nM)	447.9 ± 12.0	129.6 ± 35.4	3.5
AraC (μM)	14.7 ± 3.3	5.3 ± 0.7	2.8
5-FU (μM)	320.1 ± 11.8	155.1 ± 25.9	2.1
FUR (μM)	368.7 ± 45.4	205.3 ± 30.1	1.8
CPT (nM)	512.5 ± 20.5	671.5 ± 65.5	0.8

with radioactive gemcitabine or cytarabine for the indicated times, extracted with TCA, and the acid-soluble fraction was analyzed by HPLC with a flow radio detector. The total amounts of diphosphate and triphosphate metabolites of gemcitabine were decreased 25% and 26%, and the total amounts of diphosphate and triphosphate metabolites of cytarabine were decreased 18% and 20% in UMP/CMPK-down-regulated cells, respectively (Fig. 4).

3.4. UMP/CMPK overexpression increases sensitivity to anticancer pyrimidine analogs in the HCT8 cell line

To further study the functional role in cells, UMP/CMPK was overexpressed by plasmid transfection. Since the HCT8 cell line has a lower endogenous level of UMP/CMPK, it was chosen for an overexpression experiment. HCT8 cells were transfected with pCR3.1-UMP/CMPK, selected with G418, and an individual colony was expanded. The overexpressing lines were identified by Western blotting and an activity assay. The overexpressing lines were further cloned by serial dilution. Two clones (HCT8-K1 and -K2) with the highest levels of expression (3-fold) were chosen for further exploration (Fig. 5). UMPK, CMPK, and dCMPK activities were increased 3-fold in overexpression clones, correlating with an increased amount of UMP/CMPK protein (Table 3). Protein level and kinase activity of dCK were the same in parental and overexpressing clones. The total NDPK activity and two major cytosolic NDPK proteins, H1 and H2, were the same in parental and overexpressing clones. These results suggest UMP/CMPK overexpression will not affect the amount of protein of other activation kinases of the same pyrimidine synthesis pathway. In terms of cellular sensitivity to anticancer pyrimidine analogs, overexpressing clones were significantly more sensitive to gemcitabine (RF = 0.65 and 0.57; $p < 0.05$) and cytarabine (RF = 0.66 and 0.62; $p < 0.05$) as assayed by clonogenic assay, as expected (Fig. 6 and Table 4). The impact of overexpression was further examined in terms of pyrimidine synthesis and cell growth. We found that overexpression of UMP/CMPK has no effect on the NTP, dCTP, and TTP pool sizes, nor did it affect the cell growth rate (doubling time) and cell saturation density (data not shown). These results suggest that the indicated level of UMP/CMPK overexpression has no impact on pyrimidine triphosphate pools, DNA synthesis, or cell growth.

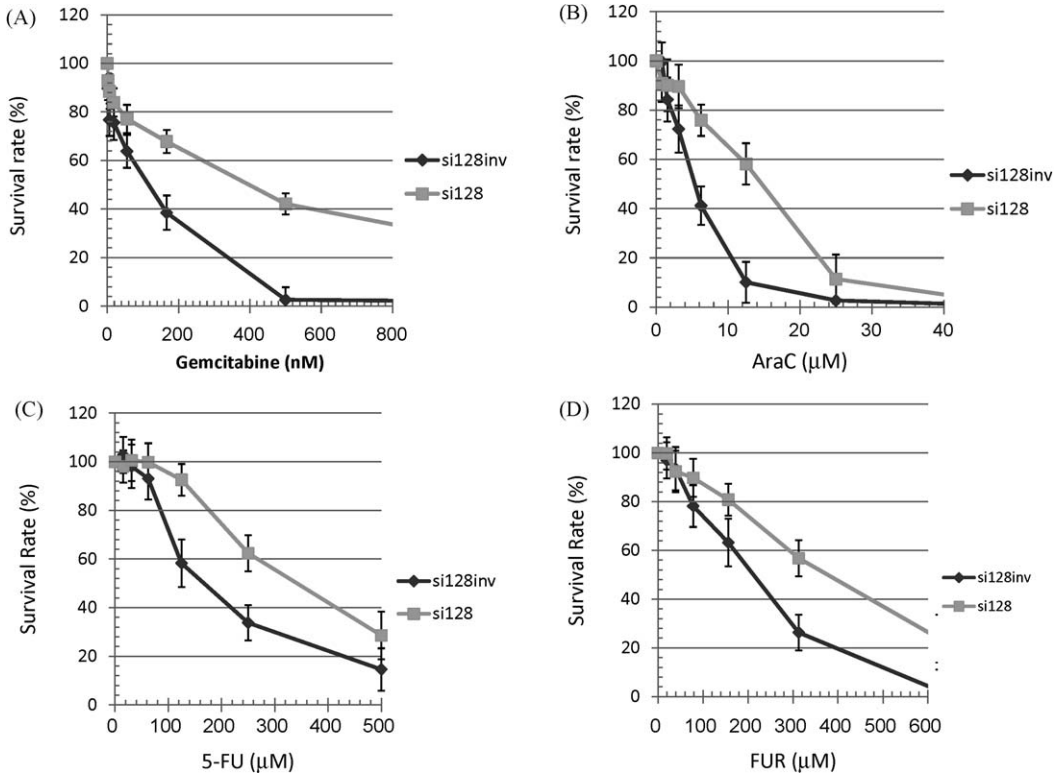

Fig. 3. Cytotoxic activity of gemcitabine (A), cytarabine (B), 5-FU (C), and FUR (D) of si128-inv and si128 siRNA-transfected HeLa S3 cells. Cells were treated with indicated agents for 4 h and clonogenic assays were performed. Points represent mean values from five independent experiments; SD is shown as an error bar.

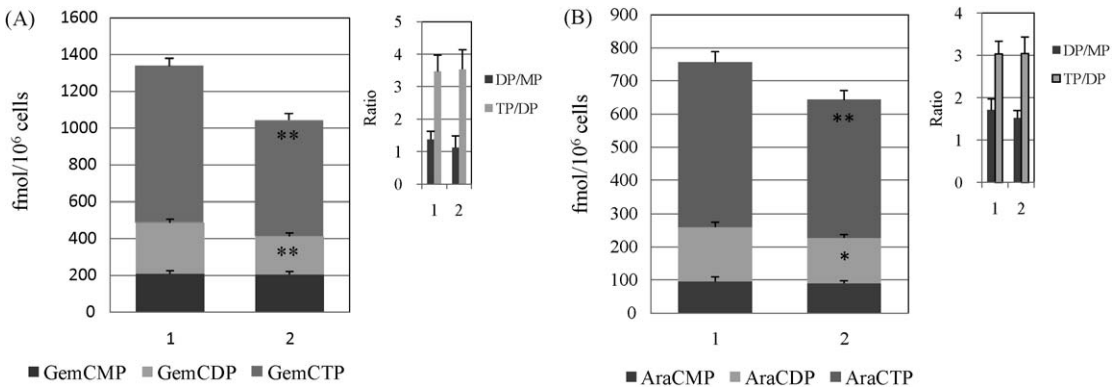

Fig. 4. Effect of UMP/CMPK down-regulation on metabolisms of gemcitabine (A) and cytarabine (B) in HeLa S3 cells. The graphs indicate the amounts of phosphorylated metabolites, and the *inset* shows the ratio of indicated metabolites. HeLa S3 cells were incubated with tritium-labeled agents for 4 h at 37 °C. The acid-soluble metabolites were extracted and analyzed by HPLC. Each bar graph represents the mean from three to five independent experiments; vertical bars, SD; $^{*}p < 0.05$; $^{**}p < 0.01$. 1 and 2 represent si128inv and si128, respectively.

3.5. UMP/CMPK overexpression increases phosphorylation of anticancer deoxycytidine monophosphate in cells

HCT8-K1 and -K2 cells are more sensitive to gemcitabine and cytarabine, suggesting UMP/CMPK overexpression may result in more gemcitabine and cytarabine phosphorylation in these cells. To test this hypothesis, control and overexpressing cells were labeled with radioactive gemcitabine or cytarabine, respectively. Both diphosphate and triphosphate metabolites of gemcitabine and cytarabine increased in UMP/CMPK-overexpressing cells (Fig. 7). These data showed that UMP/CMPK could be partly responsible for the phosphorylation of gemcitabine and cytarabine in cells; thus, the increased expression of UMP/CMPK caused cells to be more sensitive to these two agents.

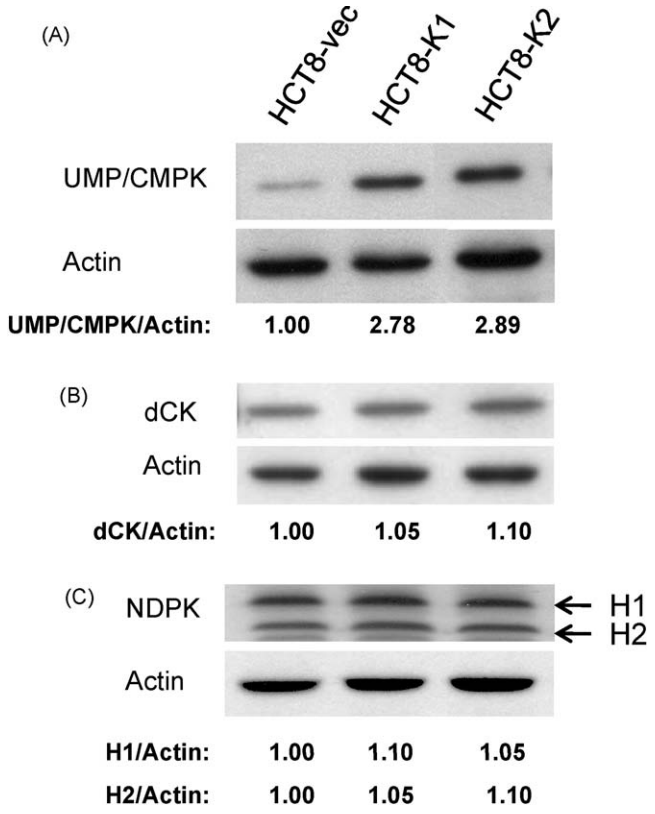

Fig. 5. Level of expression of UMPK/CMPK (A), dCK (B), and NDPK H1/H2 (C) protein in vector control and UMPK/CMPK-overexpressing HCT-8 cells. Cell lysates were prepared and analyzed by Western blotting as described. Normalized values of CMPK/actin ratio are presented.

4. Discussion

The synthesis of pyrimidine triphosphate nucleotides, in both *de novo* and salvage pathways, requires UMP/CMPK to produce diphosphates from the monophosphates. The human UMP/CMPK gene has been cloned and its enzyme activity has been characterized *in vitro*. It is a member of nucleoside monophosphate kinase family and is highly homologous to adenylate kinase [17,27]. Despite similarities in the amino acid sequence and structure, there are major differences in enzymatic characteristics among UMP/CMPKs of different species. First, although UMP/CMPKs of most eukaryotes show similar substrate specificity, yeast UMP/CMPK can phosphorylate AMP in addition to UMP and CMP [28,29]. Second, in contrast to eukaryotes in which UMP/CMPKs seem to be a single enzyme phosphorylating UMP and CMP, bacterial CMPK and UMPK are two distinctive enzymes [30–32]. Another dramatic difference between bacterial CMPK and eukaryotic UMP/CMPKs is that bacterial CMPK can phosphorylate dCMP nearly as well as CMP [29,30].

A conditional lethal mutant isolated from *Saccharomyces cerevisiae* has been identified to be caused by mutated UMP/CMPK, indicating the essentiality of this protein in the survival of this organism and possibly in mammalian cells. However, no evidence has been provided to prove its role in human cells. Similarly, anticancer pyrimidine analog monophosphates, such as Gem-, araC-, and 5-FdU-MP, have been shown to be phosphorylated by recombinant UMP/CMPK *in vitro*, and thus have been suggested to be phosphorylated by this enzyme in intact cells. However, no direct evidence has been provided and the question whether this kinase is the only enzyme responsible for the

Table 3
dCK, UMPK, CMPK, dCMPK, and NDPK activities of vector control and UMP/CMPK-overexpressing HCT-8 cells. Enzyme activity assays were performed as described. Values are presented as mean ± SD from three to five independent experiments.

	HCT8-vec	HCT8-K1	HCT8-K2
UMPK[a]	14.1 ± 1.2	34.2 ± 1.9	35.6 ± 2.1
CMPK[a]	13.3 ± 0.9	29.5 ± 2.4	30.3 ± 2.5
dCMPK[a]	9.6 ± 0.6	20.4 ± 1.8	21.2 ± 1.4
dCK[b]	62.5 ± 2.8	60.1 ± 2.5	64.3 ± 3.1
NDPK[c]	1.02 ± 0.24	0.98 ± 0.14	1.08 ± 0.18

[a] nmol/min/mg protein.
[b] pmol/min/mg protein; CdA as a substrate.
[c] unit/mg protein.

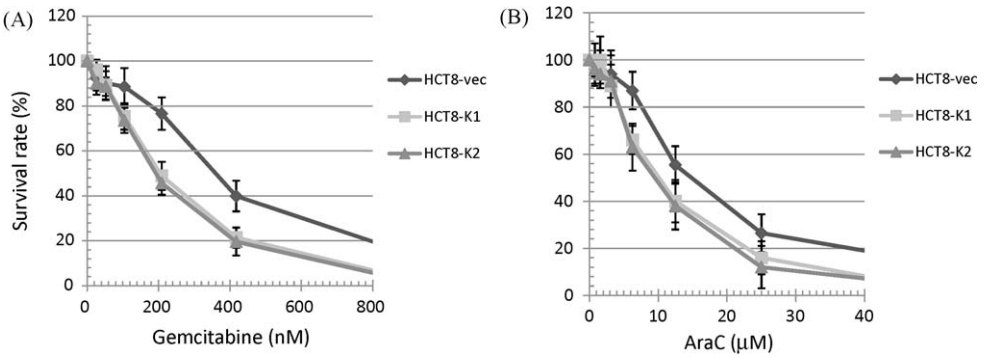

Fig. 6. Cytotoxic activity of gemcitabine (A) and cytarabine (B) in vector control and UMP/CMPK-overexpressing HCT-8 cells. Cells were treated with indicated agents for 4 h and clonogenic assays were performed as described. Points represent mean values from five independent experiments. SD is shown as error bar.

Table 4
The 50% inhibitory concentrations of gemcitabine, cytarabine, 5-FU and FUR. The data were derived from clonogenic assays. Cells were treated with indicated agents for 4 h and then cultured for 10 days before stained with methylene blue. Values represent the means ± SD of five independent experiments.

	HCT8-vec	HCT8-K1	RF (-K1)	HCT8-K2	RF (-K2)
Gem (nM)	396.8 ± 28.1	250.2 ± 24.4	0.63	219.8 ± 15.0	0.55
AraC (μM)	15.2 ± 4.2	9.9 ± 3.2	0.65	9.4 ± 2.4	0.62
5FU (μM)	183.5 ± 26.1	156.3 ± 16.2	0.85	149.5 ± 21.5	0.81
FUR (μM)	255.3 ± 30.5	210.3 ± 17.6	0.82	201.2 ± 17.2	0.79

phosphorylation of pyrimidine or its analog monophosphate to diphosphate metabolite in the cells has not been addressed.

In this study, the level of expression of UMP/CMPK RNA and protein in different cell lines was first examined. The RNA level correlated well with the protein level in the cell lines examined (correlation coefficient = 0.779; $p < 0.05$), suggesting that the difference of UMP/CMPK protein expression might be determined at the level of transcription of mRNA. This is currently under investigation.

The functional roles of UMP/CMPK in natural pyrimidine triphosphate and cell growth, as well as its impact on metabolism and cellular sensitivity to anticancer pyrimidine analogs, were studied by modulating its expression in the cells. Down-regulation of the level of UMP/CMPK protein to 20% by specific siRNA decreased UMP, CMP, and dCMP kinase activities analyzed in the cellular extract to 30%. A good correlation of the level of UMP/CMPK protein with *in vitro* activity was observed. However, the decrease did not lead to a decrease in natural pyrimidine triphosphates, such as CTP, UTP, dCTP, and TTP pools in cells, and the cell growth rate remained the same. This raises the possibility of UMP/CMPK may not be the only kinase responsible for the phosphorylation of UMP, CMP, dCMP in cytosol of the two human cell lines studied. Recently, a similar observation was also made using RKO (a human colon carcinoma cell line) cells by up- or down-regulation of UMP/CMPK (Rona et al., unpublished results).

Down-regulation of UMP/CMPK in cells specifically affected formation of the total amount of diphosphate and triphosphate metabolites of gemcitabine and cytarabine. In the case of gemcitabine, the 30% decrease in the amount of formation of diphosphate and triphosphate metabolites could lead to a decrease in the amount incorporated into the DNA. The lower expression of UMP/CMPK could have more pronounced effects on the cellular sensitivity to anticancer pyrimidine analogs. This raises the question whether this 30% decrease in analog triphosphate could account for the decrease in sensitivity of these analogs.

To further explore the role of UMP/CMPK in the metabolism of pyrimidine triphosphate and pyrimidine analog metabolism with its cytotoxicity, UMP/CMPK was overexpressed in HCT8 cells. The 3-fold overexpression of UMP/CMPK protein in HCT8 cells increased UMP, CMP, and dCMP kinase activities *in vitro* about

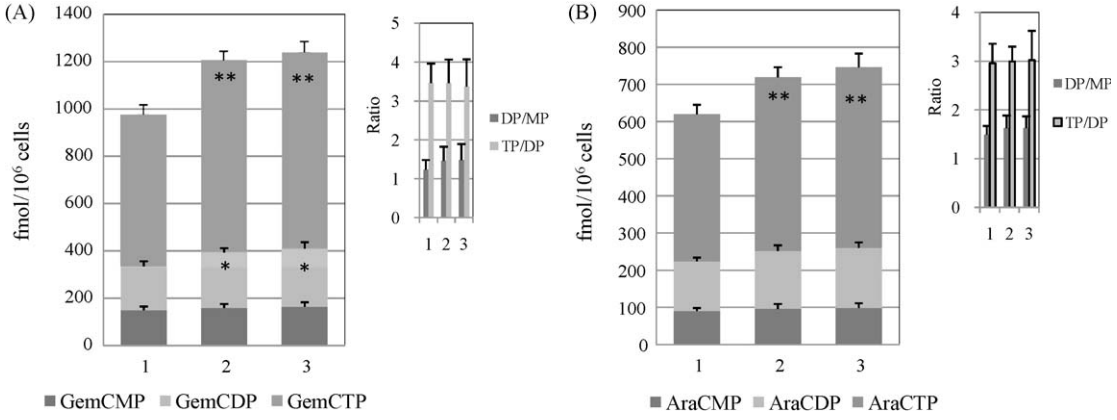

Fig. 7. Effect of UMP/CMPK overexpression on metabolism of gemcitabine (A) and cytarabine (B) in HCT-8 cells. The graphs indicate the amounts of phosphorylated metabolites, and the *inset* shows the ratio of indicated metabolites. HCT-8 cells were incubated with tritium-labeled agents for 4 h at 37 °C. The acid-soluble metabolites were extracted and analyzed by HPLC. Each bar graph represents the mean from three independent experiments; vertical bars, SD; $^{*}p < 0.05$; $^{**}p < 0.01$. 1, 2, and 3 stand for HCT8-vec, HCT8-K1, and HCT8-K2.

3-fold. However, the increase in kinase activities did not lead to higher amounts of intracellular natural pyrimidine triphosphates and increased cell growth, but could lead to increased formation of the total amount of diphosphate and triphosphate metabolites of gemcitabine and cytarabine by about 20%. This overexpression of UMP/CMPK in cells could increase cellular sensitivity to gemcitabine, cytarabine, and to a lesser extent, to 5-FU and FUR. The impact of UMP/CMPK on 5-FU and 5-FUR cytotoxicity was also observed recently by Humeniuk et al. [33,34] using HCT8 cells, but not by Rona et al. using RKO.

Based on this study and the study of Rona et al., the alteration of UMP/CMPK did not affect cellular natural pyrimidine triphosphate and cell growth. This would suggest that this protein may not be a rate-limiting protein for this metabolic process or there is another compensatory or alternative pathway for this important enzymatic process. Given the high values of Km of these monophosphates of pyrimidines and their analogs relative to the intracellular concentration of monophosphate metabolites, it is difficult to conceive that this protein is not a rate-limiting process of this metabolism if it is the only protein responsible. Recently, a novel human nucleoside monophosphate kinase, UMP-CMP kinase 2, was cloned and characterized [35]. The enzyme is able to phosphorylate dUMP, dCMP, CMP, and UMP with ATP as a phosphate donor, but the kinetic properties were different from cytosolic UMP/CMPK. The efficacy of phosphorylating dUMP is the highest among dUMP, dCMP, CMP, and UMP. A subcellular localization study showed that UMP-CMP kinase 2 was localized in the mitochondria. Surprisingly, it is claimed that the enzyme is not ubiquitously expressed in human cell lines. Whether this enzyme plays a role in the phosphorylation of UMP, CMP, and dCMP in cytosol is unclear.

In summary, UMP/CMPK may not play an important role in phosphorylating UMP, CMP, and dCMP in the cells. The level of UMP/CMPK protein correlates well with the RNA level in various human cell lines, suggesting that the regulation of expression might be at the transcription level. In HeLa S3 cells, down-regulation of UMP/CMPK expression has no impact on natural pyrimidine synthesis and cell growth, but has an impact on phosphorylation of anticancer pyrimidine analog monophosphates and cellular sensitivity to these agents. Moreover, overexpression of UMP/CMPK significantly enhances response to anticancer pyrimidine analogs in some cancer cells.

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