Aberrant Methylation of \textit{DPYD} Promoter, \textit{DPYD} Expression, and Cellular Sensitivity to 5-Fluorouracil in Cancer Cells

Takuya Noguchi, Keiji Tanimoto, Tatsushi Shimokuni, Kei Ukon, Hiroaki Tsujimoto, Masakazu Fukuishima, Tsuyoshi Noguchi, Katsunobu Kawahara, Keiko Hiyama, and Masahiko Nishiyama

Departments of 1Translational Cancer Research and 2Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; 3Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co., Ltd., Saitama, Japan; and 4Department of Surgery II, Faculty of Medicine, Oita University, Oita, Japan

ABSTRACT

\textbf{Purpose:} Dihydropyrimidine dehydrogenase (DPD), the initial rate-limiting enzyme in the degradation of 5-fluorouracil (5-FU), is known to be a principal factor in clinical responses to the anticancer agent 5-FU, and various reports have clearly demonstrated that DPD activity is closely correlated to mRNA levels. However, the regulatory mechanisms of DPD gene (\textit{DPYD}) expression remain unclear. In this study, the regulatory mechanisms have been intensively studied.

\textbf{Experimental Design and Results:} A subcloned 3.0-kb fragment of the 5’ region of \textit{DPYD} contains a total of 60 CpG sites, suggesting that methylation status may affect the repression of \textit{DPYD}. The clone showed various promoter activities that were largely correlated with mRNA levels in most cell lines, except HSC3 and HepG2. Bisulfite sequencing analysis revealed that various CpG sites around the transcription start site were abnormally methylated in cells that showed strong promoter activity. In HepG2, \textit{in vitro} methylation of the \textit{DPYD} promoter directly decreased promoter activity, and 5-azacytidine treatment restored higher \textit{DPYD} expression in a dose- and time-dependent manner, along with decreased sensitivity to 5-FU.

Conclusions: We found that DPD activity was controlled, at least in part, at the transcription level of \textit{DPYD} and that aberrant methylation of the \textit{DPYD} promoter region acted as one of the repressors of \textit{DPYD} expression and affected sensitivity to 5-FU in cancer cells. Our new results could lead to a more precise understanding of the molecular basis of 5-FU response.

INTRODUCTION

The antimetabolite 5-fluorouracil (5-FU) is one of the key agents in treatment of a large spectrum of cancers. Dihydropyrimidine dehydrogenase (DPD) is the initial rate-limiting enzyme in the degradation of 5-FU, and >80% of the administered dose of the pyrimidine analog is inactivated via this enzyme-mediated catabolic pathway; DPD is also known to be a principal factor in 5-FU pharmacokinetics, clinical toxicity, and drug resistance (1–5). The enzyme demonstrates considerable variation (8- to 21-fold) in both healthy and cancer populations: 3% to 5% of individuals have reduced DPD activity, which is associated with severe and sometimes life-threatening 5-FU toxicity in cancer patients (6). Because 5-FU accumulated in cancer cells is also rapidly converted into inactivated metabolites through catabolic pathways mediated by DPD, high DPD activity in cancer cells is an important determinant of 5-FU response (7).

This significant role of DPD in clinical 5-FU response has stimulated research aimed at prior laboratory prediction of individual response to 5-FU using DPD as a marker. The discovery that DPD deficiency is a pharmacogenetic disorder promoted the discovery of DPD gene (\textit{DPYD}) mutations that are closely linked to DPD toxicity: To date, >20 polymorphisms of \textit{DPYD} have been reported (8, 9). Among these polymorphisms, the exon 14-skipping mutation (\textit{DPYD}*2) appears to be the most prominent genetic change related to severe DPD deficiency (7). However, it is now increasingly recognized that these variant alleles are insufficient in themselves to explain either polymorphic DPD activity \textit{in vivo} or the majority (>85%) of cases of reduced DPD activity in cancer patients with 5-FU toxicity (5, 7, 10).

Because various reports have clearly demonstrated that DPD activity closely correlates to mRNA levels, recent attention has been focused on the regulatory mechanisms of \textit{DPYD} expression (10). Nevertheless, unlike the well-characterized expression profiles of \textit{DPYD} in cancer cells, the regulatory mechanisms of \textit{DPYD} expression remain unclear, and even for the sequence of the 5’ region, details are still controversial (8, 11).

In this study, we subcloned an approximately 3.0-kb \textit{DPYD} fragment that contains a total of 60 CpG sites, suggesting that methylation status may affect the repression of \textit{DPYD}. The clone showed various promoter activities that were largely correlated with mRNA levels in most cell lines, except HSC3 and HepG2. Bisulfite sequencing analysis revealed that various CpG sites around the transcription start site were abnormally methylated in cells that showed strong promoter activity. In HepG2, \textit{in vitro} methylation of the \textit{DPYD} promoter directly decreased promoter activity, and 5-azacytidine treatment restored higher \textit{DPYD} expression in a dose- and time-dependent manner, along with decreased sensitivity to 5-FU.

Conclusions: We found that DPD activity was controlled, at least in part, at the transcription level of \textit{DPYD} and that aberrant methylation of the \textit{DPYD} promoter region acted as one of the repressors of \textit{DPYD} expression and affected sensitivity to 5-FU in cancer cells. Our new results could lead to a more precise understanding of the molecular basis of 5-FU response.
we found that aberrant methylation of the DPYD promoter region could influence cellular response to 5-FU through regulation of DPYD expression.

MATERIALS AND METHODS

Chemicals. 5-FU was generously provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma (St. Louis, MO).

Cell Lines and RNA Preparation. Human cancer cell lines used were as follows: oral epidermoid carcinoma line KB (Dr. S. Akiyama; Kagoshima University, Kagoshima, Japan); colon adenocarcinoma cell line COLO201; oral squamous cell carcinoma lines HSC2, HSC3, HSC4, and Ca9-22; and hematoma cell line HepG2 (The Japanese Cancer Research Resource Bank, Tokyo, Japan). Cell lines were maintained in RPMI 1640 or Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (Bio-Whittaker, Verviers, Belgium) plus penicillin (50 IU/mL) and streptomycin (50 μg/mL) with passage every 3 days. Total RNA was prepared from frozen cell pellets by using the Qiagen RNEasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions.

Reverse Transcription-Polymerase Chain Reaction. First-strand cDNA was synthesized from 2 μg of total RNA with the use of random primer pd(N)6 and avian myeloblastosis virus reverse transcriptase XL (Takara Bio Inc., Shiga, Japan). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the TaqMan system on ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Primers and probe sequences used were as follows: DPYD1114F (forward primer, 5'-GGAGTGTGAGGATCGAGAGCGTA-3') and DPYD1232R (reverse primer, 5'-TTTGCTATGCAGTTTGTTCGGAC-3'), primer (5'-6-carboxyl-fluorescein-TTCTGCCATTCCT-GTGTCCTCATGAGGAGATGGA-3'), and probe (5'-Methylated Promoter Reporter. In vitro methylation was performed essentially as described previously (12). Briefly, pGL3-DPYDPro0.34 was digested with KpnI and XhoI, and the DPYDPro0.34 fragment was purified using the QIAquick Gel Extraction Kit (Qiagen). The purified fragment was separated into two tubes and reacted with or without 20 units of MluI-digested pGL3 basic vector at 16°C for 2 hours. The resulting fragments were purified by phenol-chloroform method and precipitated with ethanol. Purified fragments were dissolved in H2O and ligated into KpnI- and XhoI-digested pGL3 basic vector at 16°C for 2 hours. Ligated reporter constructs were purified again as described above and dissolved in 10 mmol/L Tris-HCl 10 mmol/L MgCl2, 50 mmol/L NaCl2, and 1 mmol/L dithiothreitol. The methylation reaction was optimized by confirming methylation status using bisulfite sequencing. Fragments were purified by the phenol-chloroform method and precipitated with ethanol. Purified fragments were dissolved in H2O and ligated into KpnI- and XhoI-digested pGL3 basic vector at 16°C for 2 hours. Ligated reporter constructs were purified again as described above and dissolved in 10 mmol/L Tris (pHi 8)-1 mmol/L EDTA buffer. Each DNA concentration was measured, and 0.2 or 0.4 μg of reporter with control pRL-TK was finally subjected to transfection assays as described under Luciferase Reporter Assay.

Cytotoxic Assay. Drug-induced cytotoxicity was evaluated by conventional MTT dye reduction assay. Briefly, 4 × 10³
cells were seeded in each well of 96-microwell plates (Nunclon; Nunc, Roskilde, Denmark) with regular medium. After a 24-hour incubation, the medium was replaced, and cells were exposed to various concentrations of 5-FU for 72 hours. Then, 10 μL of 0.4% MTT reagent and 0.1 mol/L sodium succinate were added to each well. After a 2-hour incubation, 150 μL of dimethyl sulfoxide were added to dissolve the purple formazan precipitate. The formazan dye was measured spectrophotometrically (570–650 nm) using MAXline microplate reader (Molecular Devices Corp., Sunnyvale, CA). The cytotoxic effect of

Fig. 1  Molecular organization of the cloned 5′ region of the DPYD gene. The 5′ region (nt –2918 to +83) of DPYD was subcloned into pGL3 basic plasmid vector and found to contain a total of 60 CpG sites, indicated as 60 bars. In these schematics, transcription start site and translation initiation site for the DPD protein are indicated as +1 and +99, respectively. Our clone contained a region consistent with the sequence reported by Shestopal et al. (11) and Collie-Duguid et al. (ref. 8; double line) and a region with a different sequence (dotted line).

Fig. 2  Relevance of endogenous DPYD expression to exogenous promoter activity in various cancer cell lines. A. Expression levels of DPYD in human cancer cell lines were analyzed by real-time RT-PCR using a TaqMan probe. Expression level normalized with GAPDH expression in each cell line is expressed relative to the level in COLO201. Exogenous promoter activity of DPYD was measured by transient transfection assay of the 5′ region of DPYD with the luciferase reporter gene. Luciferase activity normalized with pRL-TK in each cell line is expressed relative to the level in COLO201. Each group of data represents the mean ± SD for three independent experiments. B and C, correlation analyses between endogenous gene expression levels and exogenous promoter activities of the cell lines, including (B) and excluding HSC3 and HepG2 (C). Univariate analyses were carried out with StatView J.11 software (Abacus Co., Berkeley, CA).
each treatment was assessed by the IC_{50} value (inhibitory drug concentration of 50% cell growth:drug concentration of 50% absorbance of control).

RESULTS

Molecular Cloning of the 5’ Region of the DPYD Gene. Shestopal et al. (11) performed molecular cloning from a human placenta genomic library and determined functional characterization of a 1.2-kb fragment of the 5’ region of the human DPYD gene, and Collie-Duguid et al. (8) also reported the cloning of a 1.85-kb fragment as DPYD’s promoter region and its subsequent promoter activity in cultured cells. The greater parts of the suggested sequences, however, differed from each other. In our study, we first subcloned an approximately 3.0-kb fragment of the 5’ region (from nt -2918 to +83 because the transcription start site was designated as +1) of human DPYD, and we clarified the sequence (Fig. 1). The sequence analysis revealed that our clone contained a region consistent with the sequence reported by Shestopal et al. (ref. 11; from nt -1152 to +83), along with a novel upstream region as well (GenBank accession no. AB162145). As reported previously, the clone lacked a typical TATA or CCAAT box (11), and, interestingly, we found 60 CpG sites located close to the transcriptional start site in the clone.

Endogenous and Exogenous Promoter Activity of DPYD in Cancer Cell Lines. The promoter activity of the cloned DPYD was evaluated in seven human cancer cell lines using luciferase reporter analysis. Transient transfection of luciferase reporter plasmid driven by the 5’ region of DPYD demonstrated significantly different promoter activities among cell lines: activities were 6.6, 2.6, and 2.3 times stronger in HepG2, HSC2, and HSC3, respectively, than in COLO201, suggesting that promoter activity of DPYD could confer potential for DPYD gene expression (Fig. 2A). The endogenous DPYD expressions analyzed using real-time RT-PCR showed that DPYD expression also varied among cell lines, i.e., it was more than 20 times higher in HSC2 than COLO201, but undetectable in HSC3 and HepG2. Although the relative gene expression levels did not correlate with the relative exogenous promoter activities in all cell lines (Fig. 2B), they were largely correlated when HSC3 and HepG2 were excluded (\( r^2 = 0.63; \ P = 0.11 \); Fig. 2C). In HSC3 and HepG2, there was a remarkable difference between exogenous promoter activity and expression level, indicating that some factors were interfering in the regulatory mechanism. The factors responsible for this discrepancy would likely be important factors of the transcription, so we focused on the identification of these factors. One of the likely mechanisms involved in the discrepancy is a genetic mutation in the promoter region, but no differences were detected in the sequence of the 5’ region of DPYD.

Demethylation by 5-Azacytidine Treatment and Expression of DPYD. Findings in the sequence analysis showing that a total of 60 CpG sites existed in the region and that most of them were located close to the transcriptional start site led us to hypothesize that epigenetic alteration in the promoter region, i.e., aberrant methylation, might play an important role in the mechanisms of transcriptional regulation. To determine whether promoter methylation was actually participating in the transcriptional repression of DPYD in cancer cells, we performed reversal analysis of DNA methylation with 5-azacytidine treatment in HepG2. The cells were treated with vehicle, 0.25 or 2.5 \( \mu \)mol/L 5-azacytidine, for 0, 5, 10, and 20 days (13). Total RNAs were prepared from HepG2 cells in each condition, and real-time RT-PCR was performed with specific primer and probe sets for DPYD or GAPDH to clarify the influence of hypermethylation on DPYD mRNA levels. As expected, expression of DPYD was restored with 5-azacytidine treatment in a dose- and time-dependent manner, although expression of GAPDH did not change (Fig. 3A). We further analyzed expression levels of DPYD with or without 2.5 \( \mu \)mol/L 5-azacytidine treatment for 15 days in cells with undetectable (HSC3 and HepG2) and with low (COLO201) and high (HSC2) levels of DPYD: The analysis demonstrated that 5-azacytidine treatment increased DPYD expression up to 27.4 and 4.5 times in HepG2 and HSC3 but decreased it in COLO201 and HSC2 (Fig. 3B). The reverse of abnormal hypermethylation caused a remarkable increase of DPYD expression only in cells that showed extremely low (undetectable) DPYD expression; these cells, however, had potentially strong promoter activity, which led to a reduction in the initial discrepancy between expression level and promoter activity.

CpG Methylation Directly Suppressed DPYD Promoter Activity. To clarify the role of CpG methylation in DPYD promoter activity, we performed a luciferase reporter assay in
HepG2 by transfection of in vitro methylated DPYD promoter. Methylated or unmethylated DPYD promoter was ligated into pGL3 basic and transiently transfected into HepG2 cells with pRL-TK as control. In this experiment, we used a deletion mutant of the DPYD promoter reporter (pGL3-DPYDPro0.34) because an efficient religation of the 0.34-bp fragment was thought to be better than the full-length DPYD promoter clone (DPYDPro3.0). Moreover, pGL3-DPYDPro0.34 showed strong promoter activity, greater than that of full-length pGL3-DPYD-Pro3.0 in HepG2 cells (data not shown). The methylated DPYD promoter clearly demonstrated a suppressive effect on promoter activity in HepG2 cells: The promoter activity was reduced. The reduction was below 45% of the control cells that were transfected with unmethylated promoter (P < 0.0001; Fig. 4). Transfection of pRL-TK as the control did not have any effect on the results.

Demethylation by 5-Azacytidine Treatment and Cellular Sensitivity to 5-Fluorouracil. Because DPYD expression is well known to be closely correlated with DPD activity and thus with 5-FU activity against cancer cells (10), we examined cellular sensitivity to 5-FU by MTT assay with or without 5-azacytidine treatment for 15 days in HepG2. The observed IC50 value in the control cells was 2.2 µmol/L, whereas the IC50 values in cells treated with 0.25 and 2.5 µmol/L 5-azacytidine were 2.8 and 16.1 µmol/L, respectively. HepG2 showed 1.25-fold (P < 0.03) and 7.2-fold (P < 0.003) decreases in sensitivity to 5-FU with 0.25 and 2.5 µmol/L 5-azacytidine treatment, respectively (Fig. 5A). We further examined cellular sensitivity to 5-FU in other cell lines with or without 2.5 µmol/L 5-azacytidine treatment for 15 days. Demethylation caused a remarkable decrease in sensitivity to 5-FU, along with a concurrent increase of DPYD expression in HSC3, suggesting that aberrant promoter methylation affects the transcriptional repression of DPYD and thus decreases DPD-mediated 5-FU degradation in the cells.

Nevertheless, the effect of 5-azacytidine treatment on cellular sensitivity to 5-FU and DPYD expression (Fig. 5B). These results can be interpreted to mean that some peculiar mechanisms other than the DPD-mediated one are apparently involved in 5-FU response in HSC2, whereas the 5-FU response mechanisms in HepG2, HSC3, and COLO201 are DPD dependent.

Methylation Status of the CpG Island in the Core Promoter Region of DPYD. To clarify this hypothesis, the methylation status of CpG sites was studied using bisulfite sequence
Fig. 6  Methylated CpG sites in the DPYD promoter region. DNAs extracted from seven cell lines were treated with 3 mol/L sodium bisulfite for 12 hours. The 160-bp amplicons were subcloned into pGEM-T Easy vector, and 10 clones of each product were sequenced using T7 primer. The putative methylated CpG sites were then classified into three groups according to the number of residual cytosines detected in the 10 clones: 0–2, □; 3–7, ■; and 8–10, ▼. The transcription start site is indicated by an arrow.

analysis focusing on the core promoter region of DPYD, which is likely the most important in transcriptional regulation (12, 14, 15). Sodium bisulfite is a mutagen that can specifically deaminate >96% of the cytosine residues in single-stranded DNA via formation of a 5,6-dihydrocytosine-6-sulfonate intermediate. We analyzed the sequence of DNAs extracted from a total of seven cell lines, and after sodium bisulfite treatment, the 160-bp amplicons were subcloned into pGEM-T Easy vector, and 10 clones of each product were sequenced using T7 primer. As expected, residual cytosines that were unchanged after bisulfite treatment (primarily methylated cytosines) were detected at various CpG sites around the transcription start site (Fig. 6). The methylated CpG sites at −62, −34, +1, +6, and +8 were detected in cells with relatively low (KB) or undetectable (HSC3 and HepG2) DPYD expression, but were not detected in the other cell lines that had relatively high DPYD expression such as HSC2, HSC4, and Ca9-22. Among these methylated sites, the site at +8 was commonly detected in cell lines showing reduced DPYD expression, including HSC3 and HepG2 cells. However, we were unable to find any specific methylated sites in COLO201.

DISCUSSION

In this study, we looked at mechanisms that might be responsible for the expression levels of DPYD in cancer cells, and we demonstrated for the first time that DPYD promoter methylation caused DPYD repression along with an alteration of 5-FU sensitivity in cancer cells such as HSC3 and HepG2.

The relative endogenous expression levels of DPYD largely correlated with the relative exogenous promoter activities (luciferase reporter activities) in most of the cell lines tested, suggesting that transcription activities of trans-acting factors were probably critical in determining their expression levels (Fig. 7). Even so, some cells showed a discrepancy between endogenous and exogenous promoter activities. The lack of genetic variation in the 5′ region of DPYD and the existence of numerous CpG sites located close to the transcriptional start site, which was consistent with the 5′ regulatory regions of housekeeping genes and numerous tissue-specific genes (16), suggested the hypothesis that promoter methylation might play a role in repression of DPYD and could be responsible for the discrepancy between endogenous and exogenous promoter activities (refs. 12, 13, and 15; Fig. 7). In fact, our demethylation studies, transfection experiments with in vitro methylated promoter, and bisulfite sequencing analyses revealed the existence and significant role of promoter methylation. Furthermore, we observed that reversal of hypermethylation caused a decrease in sensitivity to 5-FU in HepG2 and HSC3 cells, correlated with an increase in DPYD expression. Aberrant promoter methylation of DPYD appeared to be one of the important factors in DPD-mediated 5-FU responses, through repression of DPYD.

Nevertheless, a comprehensive view of the mechanisms of DPYD transcriptional regulation is still undetermined. Although the participation of aberrant promoter methylation in these complicated mechanisms has been suggested, some of our data clearly indicated that further investigations are required. In contrast to the findings with HSC3 and HepG2, 5-azacytidine treatment increased sensitivity to 5-FU and decreased DPYD expression in COLO201 cells. Because aberrant methylation is generally understood to act on gene silencing, these results can be interpreted to mean that demethylation acts on the reversal of inhibition of transcription repressors in some cases (Fig. 7, right pathway). DPYD expression could be repressed by the mechanisms, which in turn could cause the increase in cellular sensitivity to 5-FU in COLO201 cells. However, the specific mechanism is unclear at present. Although bisulfite sequence analysis indicated that the methylation at +8 is the most likely factor associated with repression of DPYD, it is clear that large-scale investigations of methylated CpG sites in the DPYD region are necessary before any conclusion can be reached. We also need
to clarify the functional significance of all of the methylated sites and to determine whether some DPD-independent 5-FU-resistant mechanism exists. DPD-mediated degradation is not always the main mechanism of 5-FU resistance, and this was clear in the case of HSC2 in our study: Treatment with 5-azacytidine did decrease DPD expression, but at the same time, it decreased the sensitivity of HSC2 to 5-FU. Recent reports have shown that multiple genes in various types of cancer cells are heavily methylated: 5-aza-deoxycytidine treatment demethylated the hMLH1 promoter, restored its expression, and sensitized the colorectal cancer cells to 5-FU treatment (17, 18).

Fig. 7 Hypothetical model of regulation of DPD-related 5-FU response in cancer cells. DPDY is transcribed with trans-acting factors, depending on the balance between activators and repressors, and expression level of DPD correlates with its protein level and activity affecting 5-FU sensitivity. Promoter methylation may repress DPDY transcription in both activation and repression dominant status. Thus, the methylation status of DPDY could be a reliable marker for 5-FU sensitivity.

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