Deficient expression of the DPD gene is caused by epigenetic modification in biliary tract cancer cells, and induces high sensitivity to 5-FU treatment

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Received August 31, 2005; Accepted October 18, 2005

Abstract. 5-FU is the drug most frequently used to treat biliary tract cancer, while dihydropyrimidine dehydrogenase (DPD) is known to be a principal factor in 5-FU drug resistance. However, whether DPD activity and mRNA levels correlate with response to 5-FU is unknown for biliary tract cancers. The precise mechanism of DPD regulation also remains to be elucidated. In the present study, we quantitatively analyzed DPD mRNA in 8 biliary tract cancer cell lines using real-time RT-PCR, and assessed whether DPD mRNA levels correlate with DPD activity or the sensitivity to 5-FU. Finally, we examined the epigenetic gene silencing of DPD using one of the 8 lines, a gallbladder cancer cell line with deficient DPD expression, KMG-C. Strong correlation was found between DPD activity and DPD mRNA expression in the 8 cancer cell lines (R=0.797, P=0.0148). DPD mRNA expression and DPD activity exhibited positive correlation with the IC_{50} for 5-FU (R=0.658, R=0.644, respectively), although these relationships were not statistically significant. In the KMGC cells with deficient DPD mRNA levels, restoration of DPD expression was observed by 5-Aza-2' deoxycytidine (5-aza-C) treatment in a dose-dependent manner, suggesting gene suppression by promoter hypermethylation. Combined bisulfite restriction analysis was performed to analyze the methylation on CpG islands around the 5'-flanking region and intron 1 of the DPD gene, however, no methylated CpG sites were identified in these regions. In addition, the restored DPD expression level was more strongly induced by the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), than 5-aza-C treatment. These findings suggest that other mechanisms, including histone modification, may be important for DPD suppression. In conclusion, these results may aid the selection of 5-FU chemotherapy following determination of DPD expression in biliary tract cancers. Furthermore, epigenetic gene silencing appears to be an important mechanism of DPD suppression in biliary tract cancer.

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Key words: DPD, 5-FU, biliary tract cancer, methylation

Intoduction

Biliary tract cancer is highly malignant and shows a poor response against several chemotherapy agents (1-3). 5-fluorouracil (5-FU) is the most frequently administered chemotherapeutic agent for gallbladder cancer patients (4). However, in several studies, the 5-FU based regimens yielded partial responses <17% without survival prolongation (5-7). Thus, the establishment of molecular markers for 5-FU sensitivity would be a crucial step in aiding the selection of patients suitable for 5-FU chemotherapy.

Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme that metabolizes 5-FU to FUH2 (5-fluorodihydrouracil) at approximately >80% efficiency (8,9). DPD is also known to be a principal factor in 5-FU pharmacokinetics, clinical toxicity, and drug resistance. DPD activity (10,11) and DPD mRNA levels (12-14) have been reported to correlate inversely with response to 5-FU. Several reports concerning the 5-FU anti-cancer effect have indicated that DPD activity in tumors varies among various human cancer cell lines and tumors of the head and neck, liver, and colorectal cancer (10,15-17). However, there have been no findings that DPD activity, or mRNA level, correlate with response to 5-FU in biliary tract cancer.

In spite of the importance of DPD activity in 5-FU sensitivity, limited studies have been reported on the regulation mechanism of DPD expression. Several studies have demonstrated that low or deficient DPD activity is due to polymorphisms or mutations in the DPD gene. Twenty different mutations in the DPD gene have been reported, in which the most prominent mutation of the gene was a G→A mutation in the splice site of intron 14 (exon 14-skipping mutation) (18-20). However, these mutations cannot entirely explain reduced DPD activity and the toxic response to 5-FU, suggesting that a complex molecular mechanism controls polymorphic DPD activity (19,21).

Recently, DPD activity was reported to correlate with the level of DPD mRNA expression *in vitro* and *in vivo* (14,22). Furthermore, the DPD mRNA level in both colon cancer and colorectal liver metastases has been shown to be lower than in the corresponding normal tissue (23). These data indicate that DPD activity is regulated at the transcriptional level, and

several studies have examined DPD regulation. However, the precise mechanism remains to be elucidated.

In the present study, we analyzed DPD mRNA levels in 8 biliary tract cancer cell lines using real-time RT-PCR. We then assessed whether the DPD mRNA level correlates with DPD activity and the sensitivity to 5-FU. Finally, we studied the epigenetic gene silencing of DPD using a gallbladder cancer cell line with deficient DPD expression, KMG-C. We performed restoration analysis of DPD expression using the demethylation agent 5-Aza-2' deoxycytidine (5-aza-C) and the inhibitor of histone deacetylase (HDAC), trichostatin A (TSA). We also performed combined bisulfite restriction analysis (COBRA) to analyze the methylation status of the extremely GC rich sequences of the 5' flanking region and intron 1 of the DPD genome.

Materials and methods

Cell lines and culture. Eight biliary tract cancer cell lines (KMG-C, G-415, GBK-1, GB-d1, TGBC-2TKB, HBDC, HAG-1, TFK-1) were used. KMG-C, G-415, GBK-1, GB-d1 and TGBC-2TKB were obtained as described previously (24). HBDC was established in our laboratory (Jiao Wan) (25) and HAG-1 was kindly provided by Dr S. Nakano (Department of Internal Medicine, Kyusyu University, Kyusyu, Japan). TFK-1 was from the Cell Resource Center for Biomedical Research, Tohoku University (Japan). The first 7 cell lines were cultured in Williams' medium E (W/E, ICN Biomedicals INC, Costa Mesa, CA, USA), while TFK-1 was cultured in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% inactivated FBS (JRH Biosciences, Lenexa, KS, 2 mM glutamine, 100 μg/ml kanamycin and 20 μg/ml tetracycline hydrochloride. All cell lines were incubated at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Drugs. 5-fluorouracil (5-FU) was kindly supplied by Kyowa Hakko Kogyo, Co. Ltd. (Tokyo, Japan) and diluted in medium before use. 5-Aza-2' deoxycytidine (5-aza-C) was purchased from Sigma (Tokyo, Japan), and Trichostatin A was obtained from Wako (Osaka, Japan). Stock solutions of 5-aza-C and TSA were prepared at 10 mg/ml (DMSO) and 5 mg/ml (ethanol) concentrations, respectively.

Conventional reverse transcription (RT)-PCR. DPD mRNA expression was analyzed by RT-PCR as previously described (24). Briefly, total RNA was isolated from each cell line using Isogen (Nippongene, Toyama, Japan). Total RNA (1 μg) was used for first-strand cDNA synthesis. The PCR involved 30 cycles of 94˚C for 1 min, 60˚C for 30 sec, and 72˚C for 90 sec. The primers for DPD and internal marker GAPDH were as follows: DPD forward, 5'-ATG GAG GAG TGT CTG GGA CA-3'; DPD reverse, 5'-TTG AGG CCA GTG CAG TAG TC-3'; GAPDH forward, 5'-TGG TAT CGT GGA AGG ACT CAT GAC-3'; and GAPDH reverse, 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3'. The proposed sizes of the PCR products were 222 bp for DPD and 189 bp for GAPDH. All reactions were repeated at least 3 times.

Quantitative RT-PCR. To quantitatively estimate DPD mRNA expression in the 8 biliary tract cancer cell lines, real-time PCR was performed using the same primer pairs described above for conventional RT-PCR. Each cDNA after reverse transcription was also used for PCR. PCR amplification was carried out using a Light-Cycler™ system (Roche, Mannheim, Germany) and the Light-Cycler-FastStart™ DNA master SYBR green I kit (Roche). Amplifications were performed in a mixture of 1 μl cDNA, 20 pmol of primer, 4 mM $MgCl_2$ and 1 μl of Light-Cycler-FastStart DNA master SYBR green. The 3 min denaturation at 95˚C was followed by 50 cycles of 15 sec denaturation at 95˚C, 5 sec annealing at 60˚C, and 10 sec extension at 72˚C. Melting curves were then described according to the protocol with the following conditions: 0 sec denaturation period at 95˚C, starting temperature of 65˚C, ending temperature of 95˚C, and rate of temperature increase of $0.1˚s^{-1}$. The quantitative DPD values for each cell line were estimated by dividing by GAPDH expression. This analysis was performed in triplicate and the mean then calculated.

MTT assay. Drug sensitivity against 5-FU was analyzed by the MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). Cells of each line were seeded in 96-well culture plates at a density ranging from $8\sim12x10^3$ cells/well, in triplicate. After 24 h, the cells were exposed for 48 h to 5-FU at a concentration ranging from 5-200 μM. The cells were washed in PBS, fresh medium was added, and then 20 μl of MTT was added to the cultures. After a 4-h incubation, the reaction was stopped by the addition of solubilization/stop solution. Absorbance at 530 nm was measured using a multi-well plate reader (CS9300PC, Shimadzu Co. Kyoto, Japan). Data are presented as mean ± SD of 3 individual experiments.

DPD activity. DPD activity in each cell line was estimated by the ELISA method (Taiho Yakuhin Kogyo co. Ltd. Tokyo, Japan) and expressed as pmol/min/mg of protein.

Drug treatment with 5-Aza-2' deoxycytidine and Trichostatin A. To analyze restoration of DPD expression, KMG-C cells were treated with 0.4 or 2 μM 5-aza-C or 0.2 μM TSA for 24 h. RNA was extracted from the cells immediately after drug treatment and 48 h after drug treatment. Restoration of DPD expression was then assessed by RT-PCR.

Bisulfite treatment and combined bisulfite restriction analysis (COBRA). Sodium bisulfite modification of genomic DNA, followed by PCR analysis were performed as described previously (26). Briefly, 1 μg of genomic DNA was denatured with 3 M NaOH for 15 min and then incubated in solution containing 6.24 M urea, 2 M sodium bisulfite (Sigma, Tokyo, Japan) and 10 mM hydroquinone. This reaction involved 20 cycles of incubation at 95˚C for 30 sec and 55˚C for 15 min. DNA was then purified using the Gean Clean II Kit (Funakoshi, Co. Ltd., Tokyo, Japan) and desulfonated with 0.3 M NaOH. Bisulfite-treated DNA was precipitated with ethanol and resuspended in 20 μl of TE. PCR amplification was performed with 200 ng of bisulfite treated DNA, 10X Ex Taq Buffer (Takara Bio Inc, Shiga, Japan), dNTP mixture, 20 pM of each bisulfite specific primer and Takara Ex Taq HS

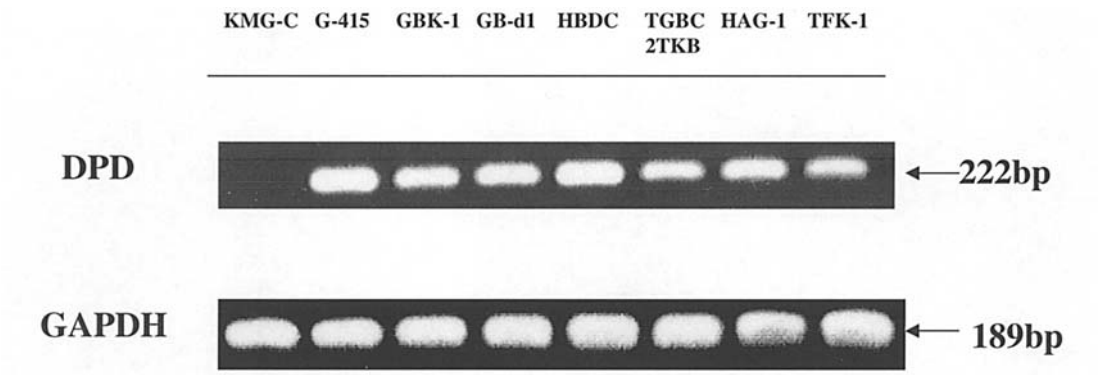

Figure 1. DPD mRNA expression in 8 biliary tract cancer cell lines by RT-PCR analysis. DPD mRNA expression was lacking in KMGC, although it was observed in the other cell lines.

A

	DPD activity	DPD mRNA/GAPDH
KMGC	0	0.00155
GB-d1	29.9	0.0449
HBDC	62.5	0.0729
GBK-1	13.2	0.0245
G-415	90.2	0.1423
TGBC	64.9	0.0437
HAG1	13.8	0.0645
TFK1	18.8	0.0146

B

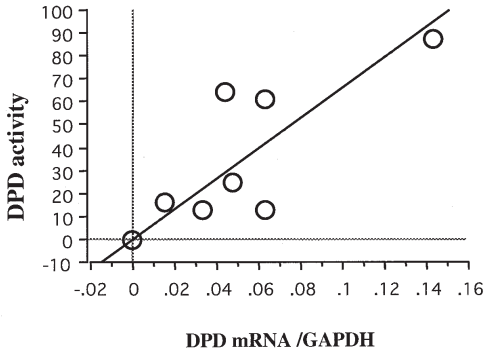

Figure 2. The value of DPD mRNA and DPD activity derived from quanititative RT-PCR and ELISA, respectively (A). The correlation between DPD mRNA and DPD activity was analyzed by statview software (B). A strong correlation was found between DPD activity and DPD mRNA expression in 8 cancer cell lines (R=0.797, P=0.0148). The quantitative DPD value in each cell line was estimated by dividing with GAPDH expression.

(Takara Bio Inc, Shiga, Japan). This PCR was composed of 40 cycles of 94˚C for 1 min, 58˚C for 30 sec and 72˚C for 90 sec. The bisulfite specific primers were as follows: bisulfite 1F, 5'-TGT TTG GGG ATT TTT TTT ATT TGG T-3'; bisulfite 1R, 5'-CAC AAA AAC CAT AAC AAT ACC TAC AA-3'; bisulfite 2F, 5'-TGG TTT TTG TGT TTA GTA AGG ATT-3'; and bisulfite 2R, 5'-CAA TCA CTA ACA TTC AAA AAA AAA CA-3'. The PCR products were digested with Taq1 for 90 min at 65˚C and ACC for 90 min at 37˚C. The resultant DNA fragments were then subjected to 2% agarose gel electrophoresis and stained with ethidium bromide.

Bisulfite sequencing. The PCR products from bisulfite treated DNA were subcloned into the pSTBlue-1 TA cloning vector system (Novagen, Inc., Darmstadt, Germany). After transformation in competent *E. coli* DH5α cells, plasmid DNA from 4 clones was prepared. DNA sequence analysis of each of the 4 clones was performed using an automated DNA sequencer (ABI PRISM 310, Perkin-Elmer Applied Biosystems Division, Foster City, CA).

Statistical analysis. The relationships among IC_{50} for 5-FU, DPD activity and DPD mRNA expression from the 8 biliary tract cancer cell lines were assessed statistically by Pearson's correlation using Statview software.

Results

DPD expression among the 8 biliary tract cancer cell lines. Fig. 1 shows the DPD mRNA expression in the 8 biliary tract cancer cell lines. Seven cell lines, G-415, GBK-1, GB-d1, HBDC, TGBC-2TKB, HAG-1 and TFK-1 expressed DPD mRNA, however no DPD expression was found in the KMG-C cells.

DPD activity and DPD mRNA expression. The expression of DPD mRNA in the 8 biliary tract cancer cell lines was quantitatively assessed by real-time PCR and compared with the DPD activities (Fig. 2A). The DPD activity of KMG-C was extremely low and was below the threshold level of the assay, as was mRNA expression (0.00155). DPD activity and mRNA level in G-415 was the highest among the 8 cell lines. Strong correlation was found between DPD activity and DPD mRNA expression for all 8 cancer cell lines (R=0.797, P=0.0148) (Fig. 2B).

Sensitivity to 5-FU in the 8 biliary tract cancer cell lines. Drug sensitivity to 5-FU was assessed by the MTT assay. The IC_{50} for 5-FU for each of the 8 cell lines is shown in Fig. 3. KMG-C, with deficient DPD expression, was significantly sensitive to 5-FU. In contrast, G-415, with high DPD activity and mRNA

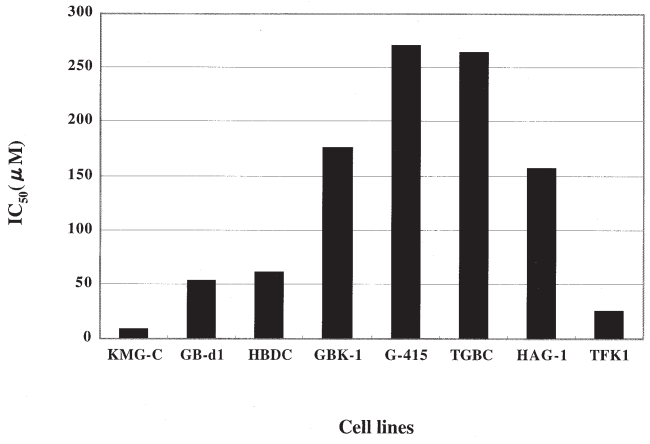

Figure 3. IC_{50} for 5-FU in 8 biliary tract cancer cell lines was estimated by MTT assay. MTT assays were performed in triplicate measurements. IC_{50} values were calculated and the mean of three individual experiments was plotted.

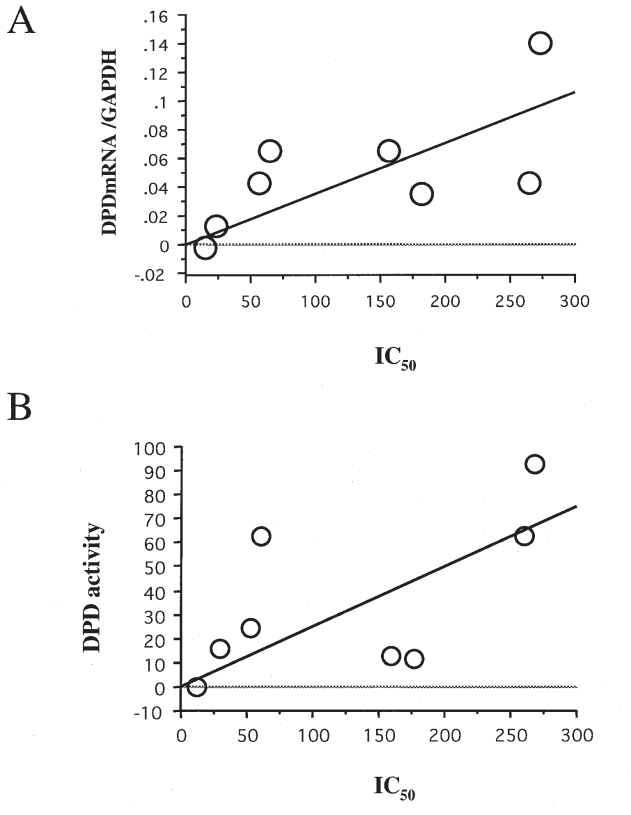

Figure 4. The relationship between DPD mRNA and IC_{50} for 5-FU in 8 biliary tract cancer cell lines (A). The relationship between DPD activity and IC_{50} for 5-FU in 8 biliary tract cancer cell lines (B). Positive correlation between DPD mRNA, DPD activity and IC_{50} for 5-FU were observed (R=0.658, R=0.644, respectively; P=0.0776, P=0.0875 respectively).

expression, was the most resistant among the 8 cancer cell lines. The IC_{50} for G-415 was 29.7-fold higher than that of KMG-C. Fig. 4 shows the relationship between DPD mRNA expression or DPD activity and the IC_{50} for 5-FU, respectively. DPD mRNA expression or DPD activity and the IC_{50} for 5-FU exhibited positive correlation (R=0.658, R=0.644, respectively), although these relationships were not statistically significant.

The restoration of DPD expression by 5-azacytidine and TSA treatment. According to the sequence of DPD genomic DNA, a GC rich region exists around the transcription start site (GC content is 68.7% from -268 to +113) and first intron (67.7% GC from +103 to +440) (Fig. 5). Thus, DPD expression may be suppressed by epigenetic gene silencing. To determine whether this is true, restoration analyses using a methyltransferase inhibitor, 5-aza-C, and a histone deacetylase inhibitor, TSA, were performed in the KMG-C cell line, which lacks DPD expression. DPD expression was not restored by 24-h treatment with 0.4 μM 5-aza-C, however, expression was restored by 2 μM treatment (Fig. 6A). At 48 h after treatment, DPD expression was restored even by 0.4 μM 5-aza-C, and stronger restoration was also found by 2 μM exposure for 48 h (Fig. 6B). TSA treatment after both 0 h and 48 h restored DPD expression more strongly than 2 μM 5-aza-C exposure.

COBRA analysis. To examine methylation of the DPD promoter, COBRA was performed using a bisulfite specific primer that covered the region around the transcription start site, as described in Fig. 5. Methylation specific digestion by both Taq1 and ACCII was not seen spanning -268 to +113 of the DPD genome in KMG-C cells, whereas digestion signals by these restriction enzymes were found in the positive control DNA (Fig. 7A). We then performed COBRA using a DNA fragment spanning exon 1 (+103) to intron 1 (+440), however, no methylation signal was detected (Fig. 7B).

Discussion

In the present study, we examined the DPD mRNA levels among 8 biliary tract cancer cell lines by quantitative RT-PCR and compared the findings with the IC_{50} for 5-FU. In the last decade, several studies of DPD expression and 5-FU sensitivity have been reported in gastrointestinal cancers (27,28). In the present study, we provide evidence that DPD mRNA expression and DPD activity are correlated with the IC_{50} for 5-FU in biliary tract cancer, indicating transcriptional regulation of DPD expression. Moreover, we identified a DPD deficient gallbladder cancer cell line among the 8 used, namely KMG-C, which exhibited the highest sensitivity to 5-FU. Various lengths of the 5' flanking region of the DPD gene have been cloned and sequenced by several researchers (18,29,30). The DPD gene lacks the typical TATA or CCAAT boxes, instead having several GC rich regions, around the transcription start site. Numerous studies have demonstrated cancer specific gene silencing due to an epigenetic mechanism, by which cytosine residues on a CpG sequence on the gene promoter were highly methylated (31-33).

Thus, we concluded that epigenetic modification of the DPD promoter region might contribute to DPD regulation at the transcriptional level. We demonstrated that 5-aza-C restores DPD expression in a dose- and time-dependent manner. This finding raises the possibility that promoter methylation plays a crucial role in the down regulation of DPD expression. Using oral squamous and hepatocellular cancer cell lines, Noguchi *et al* identified several methylated CpG sites around the transcription start site (-62 - +13) of the DPD promoter (30).

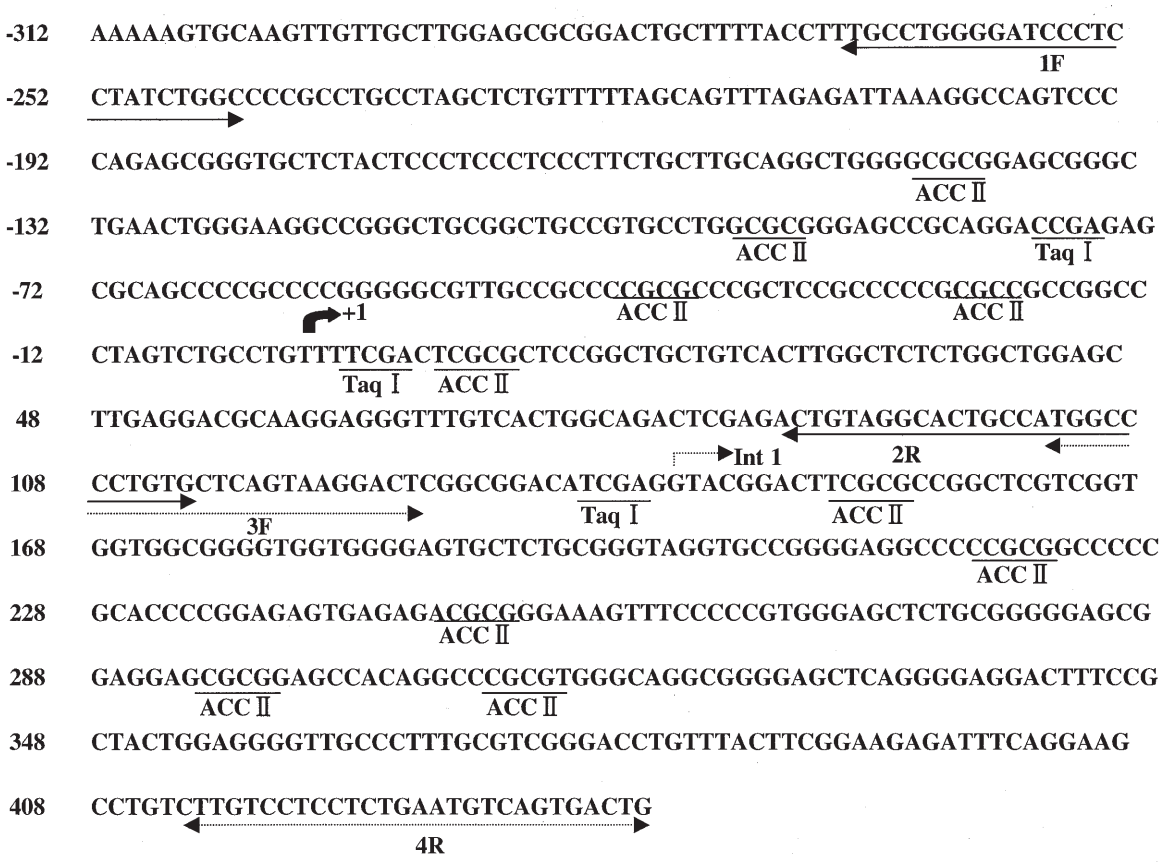

Figure 5. The sequence of DPD gene (-312 - +440) around the transcription start site. The transcription start site is described as +1. Bisulfite primer 1F-2R is shown by bold arrow and 3F-4R is by dotted arrow. The restriction sites of ACCII and Taq1 used in COBRA are underlined. Start site of intron 1 is marked as Int 1.

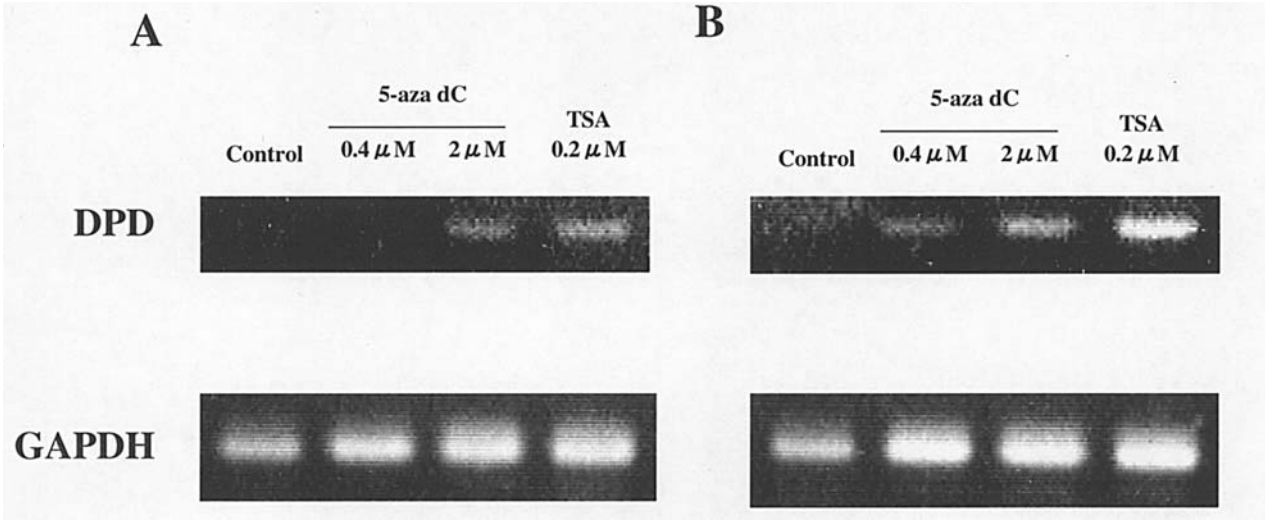

Figure 6. The restoration of DPD mRNA expression by methyltransferase inhibitor, 5-aza-C and histone deacetylase inhibitor, TSA in KMG-C lacking DPD expression. (A), The result at 0 h after 24-h treatment by 5-aza-C and TSA. (B), The result at 48 h after 24-h treatment by by 5-aza-C and TSA.

However, in the present study, COBRA using KMG-C cells did not detect methylated cytosine around the transcriptional start site (-268 - +113) nor the region spanning exon 1 to intron 1 (+103 - +441). We further performed bisulfite sequence analysis to investigate cytosine methylation at all CpG sites, however no methylated cytosines were detected in these two GC rich regions (data not shown). These results suggest that silencing of DPD expression in KMG-C cells is most likely not caused by promoter methylation. In fact, we found that TSA treatment restored DPD mRNA expression more strongly than 5-aza-C treatment of KMG-C cells. This finding suggests that another epigenetic gene silencing mechanism, such as histone deacetylation, may be implicated in DPD suppression (34-36). DNA methylation and histone

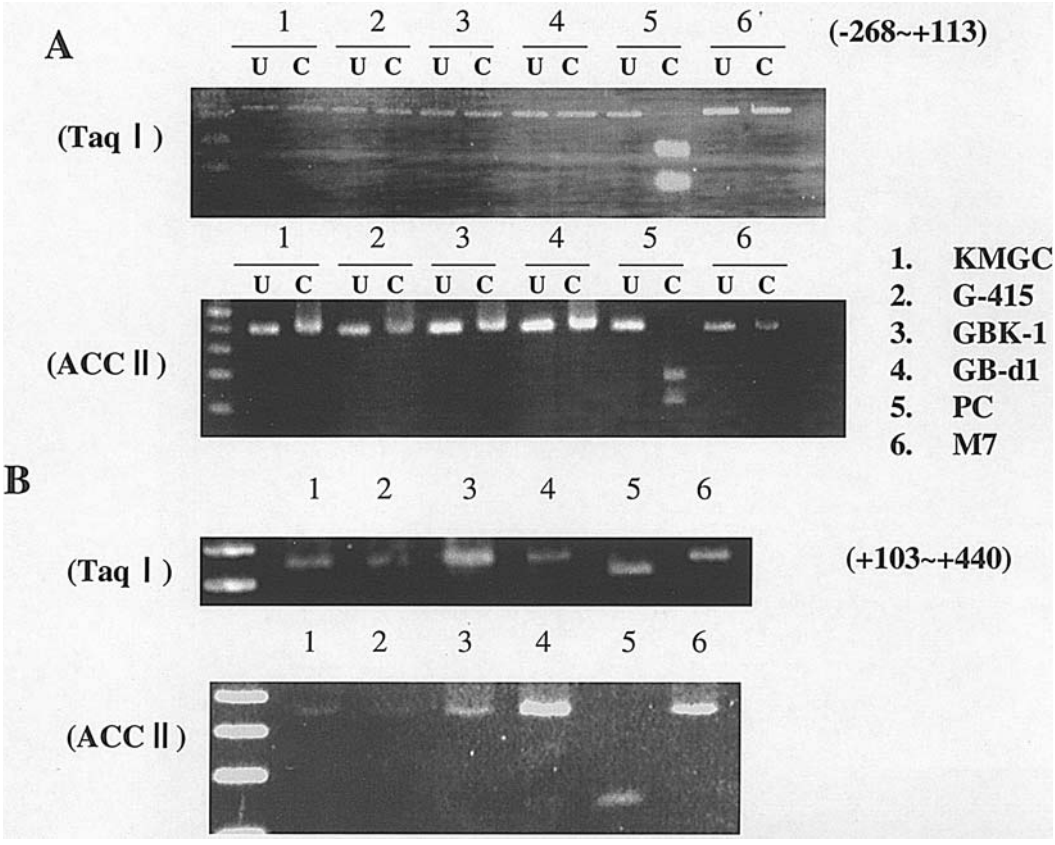

Figure 7. The result of COBRA using DNA fragment around the transcriptional start site (-268 - +113) (A) or exon 1 - intron 1 (+103 - +441) (B) are presented. Taq I and ACCII were used as bisulfite specific restriction enzymes, respectively. PC:methylated DNA template for positive control. M7:unmethylated DNA template for negative control.

modification are known as the major mechanisms of epigenetic gene silencing, and close linkage between these two regulatory processes has been reported (34,37,38). However, Bachman *et al* have reported that histone modification associated with silencing of a tumor suppressor gene can occur independently of DNA methylation (39). These reports support our hypothesis that deficient DPD expression in KMG-C might be caused not by cytosine methylation, but by histone modification.

In conclusion, we have demonstrated that both DPD mRNA expression and DPD activity are correlated with the sensitivity to 5-FU in biliary tract cancer cell lines. Furthermore, a close relationship was observed between DPD mRNA expression and DPD activity. These findings may aid the selection of 5-FU chemotherapy against biliary tract cancers. Furthermore, the present study implies that inhibition of DPD expression may be regulated by epigenetic alteration such as histone modification. Further investigations are required to clarify the precise mechanism of DPD transcriptional repression.

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