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Research Article

Sorafenib Decreases Dihydropyrimidine Dehydrogenase Gene Expression in Hepatocellular Carcinoma HepG2 Cells

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Abstract

Aim: Sorafenib, a multi-kinase inhibitor, is the only first-line oral drug for patients with advanced hepatocellular carcinoma (HCC). We recently reported that sorafenib enhances the anti-tumor effects of hepatic arterial infusion chemotherapy (HAIC) using 5-Fluorouracil (5-FU) against HCC. However, the mechanism by which sorafenib increases sensitivity to chemotherapy remains unknown. Herein, using hepatocellular carcinoma HepG2 cells, we investigated the mechanism by which sorafenib regulates 5-FU sensitivity.

Methods: The expression of genes of interest was assessed by DNA microarray, real-time PCR, western blot, and dual luciferase assay.

Results: Dihydropyrimidine dehydrogenase (DPYD) gene, a key enzyme in 5-FU inactivation, was down-regulated by sorafenib. Real-time PCR, western blot, and dual-luciferase assays indicated that DPYD mRNA and protein expression as well as its promoter activity were decreased by sorafenib in HepG2 cells. Furthermore, DPYD promoter activity was decreased by sorafenib via the Sp1/3C site on the 5′-flanking site of DPYD.

Conclusion: Our results indicate that sorafenib decreases DPYD expression in HepG2 cells.

Keywords: 5-fluorouracil; Dihydropyrimidine dehydrogenase; HepG2; Sp1/3; Sorafenib

Introduction

Hepatocellular carcinoma (HCC), a disease often caused by hepatitis B or C or cirrhosis, is the sixth leading cause of death from cancer, worldwide [1]. Although a small percentage of patients are diagnosed at an early stage, most patients go undiagnosed until the disease has progressed to advanced HCC (aHCC), for which available therapies are hardly effective. Hepatic arterial infusion chemotherapy (HAIC) with 5-fuorouracil and cisplatin using an infusion pump and implanted reservoir prolongs survival and improves the quality of life of patients with aHCC [2]. Sorafenib, a multi-kinase inhibitor, is the only used oral molecular targeted drug for patients with aHCC. Sorafenib is not only approved for the treatment of aHCC, but also for that of primary kidney cancer and advanced thyroid carcinoma. Sorafenib affects cancerous cells and tumor-associated endothelial cells. Sorafenib inhibits rapidly accelerated fibrosarcoma (Raf) kinase, thereby suppressing the proliferation of cancerous cells via inhibition of the mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK kinase, MEK)-ERK pathway [3]. Sorafenib also inhibits the tyrosine kinase activity of platelet-derived growth factor receptor (PDGFR)- and vascular endothelial growth factor receptor (VEGFR)-2 and -3. Therefore, sorafenib inhibits angiogenesis in tumor-associated endothelial cells, thereby suppressing tumor vessel growth [4]. Effects of sorafenib on apoptosis-related events have been reported [5,6]. According to Ullen et al., sorafenib induces not only apoptosis, but also autophagy in prostate cancer cells [7]. In addition, sorafenib presents other functions such as changes of cytokine levels [8] and inhibition of hypoxia-inducible factor (HIF)-1α synthesis [9].

We previously described that pre-administration of sorafenib before HAIC significantly extended the survival of patients with HCC [10]. However, the mechanism by with sorafenib sensitizes HCC cancer cells to chemotherapy, such as HAIC with 5-FU, remains unknown. The present study was undertaken to investigate the effect of sorafenib on the regulation of genes of interest and the metabolism and transport of 5-FU using hepatocellular carcinoma HepG2 cells in vitro.

Materials and Methods

Materials

U0126 was purchased from Promega (Madison, WI, USA). Sorafenib was purchased from Chem Scene (Monmouth Junction, NJ, USA).

Cell culture

The human hepatocellular carcinoma cell line, HepG2, was purchased from Japanese Cancer Research Resources Cell Bank (JCRRB, Tokyo, Japan). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (WAKO, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and 10mg/L of streptomycin sulfate, 10,000units/L of penicillin G and 250μg/L Amphotericin B in a

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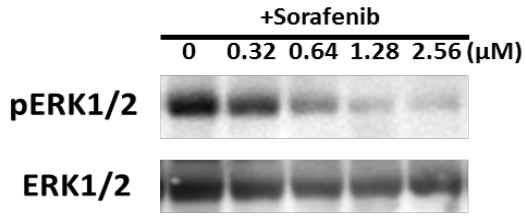

Figure 1: Effect of sorafenib on ERK phosphorylation in HepG2 cells. After HepG2 cells were treated with 0–2.56 μmol/L sorafenib for 48h, the cells was harvested and lysed. Extracted proteins were electrophoresed by 12% SDS-PAGE and transferred to PVDF membrane. The levels of ERK and phosphorylated ERK were determined by western blot using specific antibodies and POD-conjugated secondary antibodies.

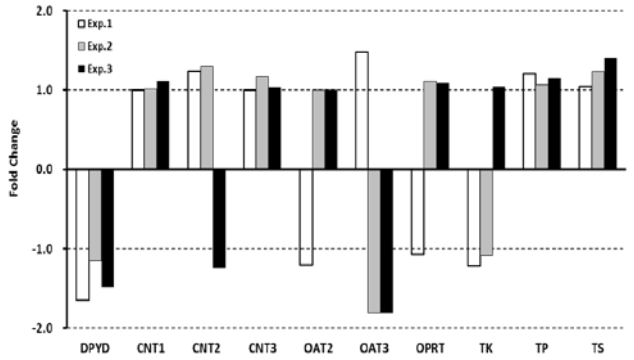

Figure 2: Changes in mRNA expression of 5-FU-related transporters and enzymes in HepG2 cells treated with sorafenib by microarray analysis. After HepG2 cells were treated with 0.32 μmol/L sorafenib for 48h, total RNA was extracted from HepG2 cells and labeled-cRNA was obtained from cDNA. mRNA levels were determined by microarray analysis using Sure Print G3 Human GE 8x60K Ver.2.0 microarray and microarray scanner.
DPYD: Dihydropyrimidine Dehydrogenase; CNT: Concentrative Nucleoside Transporter; OAT: Organic Anion Transporter; OPRT: Orotate Phosphoribosyl Transferase; TK: Thymidine Kinase; TP: Thymidine Phosphorylase; TS: Thymidylate Synthetase.

humidified atmosphere containing 5% CO_2 at 37°C.

Phosphorylation status of ERK

To determine the suitable concentration of sorafenib to be used in the subsequent assays, ERK phosphorylation was assessed using western blotting (see details in the corresponding section below).

Total RNA extraction

Total RNA was extracted from HepG2 cells treated with 0-1.28 μmol/L sorafenib or 0-12.5 μmol/L U0126 using ReliaPrep™ RNA Cell Miniprep Systems (Promega) according to the manufacturer's instructions. The RNA concentration and A260/A280 ratio were determined by a Smart Spec Plus spectrophotometer (BIORAD, Hercules, CA, USA), and the 28S/18S ratio was analyzed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) for microarray analysis. The total RNA samples that presented an A260/A280 ratio >1.8 and 28S/18S ratio >1.80 were used for microarray and real-time PCR analysis.

Microarray analysis

The first-strand cDNA, transcription, and labeling of cRNA were performed by using 50ng of total RNA and Low Input Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. The labeled cRNA was purified using RN easy mini spin column (QIAGEN, Valencia, CA, USA) and hybridized on SurePrint G3 Human GE 8x60K Ver.2.0 microarray (Agilent Technologies). The data were obtained by using Agilent Feature Extraction software ver. 10.7.3.1 (Agilent) after the array slide was scanned by Microarray Scanner (Agilent Technologies).

Real-time PCR

The cDNA was synthesized from 1μg of total RNA from sorafenib-treated HepG2 cells using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. cDNA (0.5μL) was amplified in a ABI PRISMR 7500 Fast (Life Technologies Corporation, Carlsbad, CA, USA) using THUNDERBIRD® Probe qPCR Mix (TOYOBO) and specific primer sets according to the manufacturer's instructions. PCR conditions were as follows: 95°C for 5min, followed by 40 cycles of 95°C for 15s and 60°C for 60 s. Specific primer sets used were TaqMan® Gene Expression Assay (Applied biosystems) ID#Hs00559279_m1 for DPYD and ID#Hs99999903_m1 for β-actin. Gene expression was normalized to that of β-actin and analyzed by using the comparative Ct method ($2^{-\Delta\Delta}$Ct).

Western blotting

After treatment with 0–2.56 μM sorafenib at 37°C for 48h, the HepG2 cells (3mL, 5 × 105 cells/mL) were lysed by sonication. The lysate was centrifuged at 2,000 x g for 15min and the supernatant was separated on 12% SDS-PAGE. After transfer to a Hybond-P PVDF membrane (GE Healthcare Bio-Science, Uppsala, Sweden), the membrane was blocked with PVDF Blocking Reagent (TOYOBO) at 4°C overnight and probed with 0.5μg/mL anti-DPYD mouse antibody (Abnova Corp., Taipei, Taiwan), 0.5μg/mL anti-β-Actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-ERK1/2 (EnoGene, New York, NY, USA), and anti-phosphorylated ERK1/2 (EnoGene) in Can Get Signal Solution-I (TOYOBO) for 1h at 20 degree, followed by further incubation with 0.025μg/mL HRP-labeled anti-mouse IgG goat antibody (Kirkegaard & Perry Laboratories Inc., Milford, MA, USA) or HRP-labeled anti-rabbit IgG (H+L) goat antibody (Kirkegaard & Perry Laboratories) in Can Get Signal Solution-II (TOYOBO) for 1h at room temperature. The membrane was then washed 3 times with PBS-T and the proteins were incubated with ECL-plus (GE Healthcare Bio-Science) for 5min at room temperature and analyzed with a Typhoon 9410 (GE Healthcare Bio-Science). Densitometric analysis was performed using Image Quant ver. 5.0 (GE Healthcare Bio-Science). The signals were normalized to that of β-actin using Image J software (ver. 1.47V).

Reporter gene assay for DPYD promoter

Genomic DNA from HepG2 cells (1 × 105 cells) was extracted with the Wizard SV Genomic DNA preparation system (Promega). DPYD promoter construct containing nt -836 to +120 from the transcription initiation site was cloned by PCR from genomic DNA using the KOD FX (TOYOBO) containing 20% of PCR Enhancer (Life Technology) and specific primers, 5-CAAGCAGGCATCACATTTTC-3′ (sense) and 5′-CCAGTGACAAACCCTCCTTG-3′ (antisense). PCR products were 5′-phosphorylated using T4 Polynucleotide Kinase (TOYOBO) and then ligated into the pGL4.11 vector that had been digested with EcoRV (TOYOBO) and treated with alkaline phosphatase from *E.*

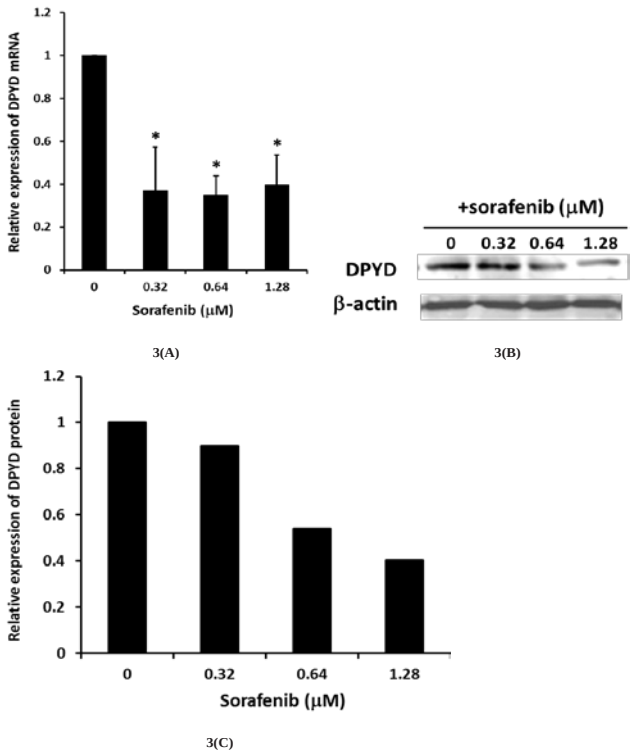

Figure 3: DPYD mRNA and protein expression in HepG2 treated with sorafenib. (A): *DPYD* mRNA expression levels were determined by real-time RT-PCR. Results were obtained from three independent experiments and represent mean ± SD normalized to β-actin. (B): HepG2 cells were treated with 0–1.28 μmol/L of sorafenib for 48h. DPYD protein levels in the HepG2 cells were determined by western blotting. Proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane. DPYD and β-actin protein levels were determined by using anti-DPYD and anti-β-actin and POD-conjugated secondary antibodies. (C): Densitometric analysis of DPYD protein expression assessed by western blot.
Each value is shown relative to that of control HepG2 cells. Asterisks indicate significant differences ($P< 0.05$) as compared with HepG2 cells.

coli (TOYOBO). The 5′ regions were then sequenced using a 3730 x l DNA analyzer (Life Technology). Reporter plasmids (2μg each) were transfected into HepG2 cells (10 × 104 cells) with pGL4.73 (pRL-SV40, Promega) (0.1μg) using Lipofectamine 2000 (Life Technology). Eight hours after transfection, HepG2 cells were treated with or without 0.64μmol/L sorafenib or 0-12.5 μmol/L U0126 for 16h, and luciferase activities were determined by a 20/20n luminometer (Promega) using a Dual Luciferase Assay Kit (Promega). Luciferase activities of reporter plasmids were normalized to those of SV40-Luc activity in transfected cells.

Mutagenesis of DPYD promoter regions

Site-directed mutagenesis of the *DPYD* promoter was carried out by using KOD mutagenesis kit (TOYOBO). The pGL4.11/-836_+120 plasmid were used as a template. The Sp1 mutant sequences were reported by Xue [11]. The mutated primers used for site directed mutagenesis were 5′-A A A C T G T A T G C T G C T G C G G G C T G T-3′ and 5′-T G C T G C A G G T G G T A C A T A A T C A A G-3′ for elk-1, 5′-A A T G C G G A G C G G G C T G A A C T G G G A A G G C C G-3′ and 5′-C C A G C C T G C A A G C A G A A G G G A G G-3′ for Sp1A, 5′-T G T T C C G G G G G C G T T G C C G C C C C G C-3′ and 5-G G G C T G C G C T C T C G G T C C T G C G G C T-3 for Sp1B, and 5′-T G T T C C C G C G C C G C C G G C C C T A G T C T G C C T-3′ and 5′-G A G C G G G C G C G G G G C G G C A A C G C C C-3′ for Sp1C mutants (underlined nucleotides indicate the mutation sites). Inversed PCR, *DpnI* digestion, ligation, and transformation were performed according to the manufacturer's instructions. The mutated plasmids were confirmed by DNA sequencing using a 3730 x l DNA analyzer (Life Technology).

Statistical analysis

All experimental data are presented as mean ± SD and were analyzed by Student's t-test. Statistical significance was defined as a *P* value less than 5%.

Results and Discussion

Sorafenib decreases ERK phosphorylation

Sorafenib is a potent inhibitor of RAF kinase and the RAF-MEK-ERK cascade in HCC cells [12]. To determine how sorafenib regulates gene expression in HepG2 cells, we determined the suitable concentration of sorafenib by assessing ERK phosphorylation as an indicator. ERK phosphorylation decreased in a dose dependent manner by 8h of treatment with 0–1.28 μmol/L sorafenib (Figure 1).

Microarray analysis for 5-FU metabolism related gene

We determined the expression of genes of interest to determine how sorafenib regulates gene expression [13]. The genes that function as transporters of sorafenib were concentrative nucleoside transporter (CNT) 1-3 and organic anion transporters (OATs), and those involved in 5-FU metabolism were and dihydropyrimidine dehydrogenase (DPYD), thymidine phosphorylase (TP), thymidine kinase (TK), thymidylate synthetase (TS), and orotate phosphoribosyl transferase (OPRT) (Figure 2). We treated HepG2 cells with 0.32μmol/L sorafenib, a non-toxic dose, for 16h and studied the mRNA expression of these genes by cDNA microarray. Of all these genes, sorafenib only affected the expression of DPYD, which was significantly decreased ($P< 0.05$). Therefore, we focused on the effects of sorafenib on DPYD expression.

Sorafenib decreases DPYD mRNA and protein levels

To investigate whether DPYD mRNA expression was decreased by sorafenib treatment, we quantitatively determined DPYD mRNA levels. After HepG2 cells were cultured in the presence of 0–1.28 μmol/L sorafenib for 16h, DPYD mRNA expression levels were determined by real-time PCR using gene specific probes. As shown in Figure 3A, DPYD mRNA expression was suppressed by sorafenib. DPYD protein levels were also decreased by sorafenib treatment (Figure 3B). DPYD protein levels were determined by densitometric analysis (Figure 3C) and were significantly decreased in HepG2 cells treated with more than 0.64μmol/L sorafenib.

Effect of MEK inhibitor (U0126) on mRNA expression and promoter activity of DPYD

Although sorafenib does not directly inhibit MEK-1 or ERK-1 kinase activity, sorafenib inhibits Raf serine/threonine kinase-1 (Raf-1) in tumor cells, thereby deregulating MEK and ERK signaling [12,14]. To clarify whether inhibition of MEK/ERK signaling can decrease DPYD expression, we treated HepG2 cells with U0126 (MEK-1 inhibitor) for 16h. These cells were harvested and DPYD

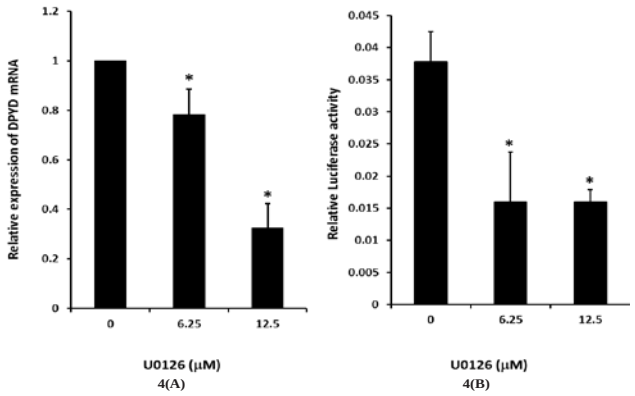

Figure 4: mRNA levels and promoter activity of DPYD gene in HepG2 cells in the presence of an MEK inhibitor, U0126. (A): HepG2 cells were treated with U0126 (0–12.5 μmol/L) for 16h at 37°C. *DPYD* mRNA expression levels were determined by real time RT-PCR. Expression levels were normalized to those in cells treated without inhibitors and considered as 1. The results are expressed as mean ± SD, obtained from three independent experiments. Asterisks indicate significant differences (*P*< 0.05). (B): HepG2 cells were treated with U0126 (0–12.5 μmol/L) for 16h at 37°C after co-transfection of 2μg of the plasmid construct (pGL4.11/-836_+120) and 0.1μg of pRL-SV40. The results from three independent experiments are expressed as mean ± SD. Asterisks indicate significant differences (*P*< 0.05) as compared with untreated HepG2 cells.

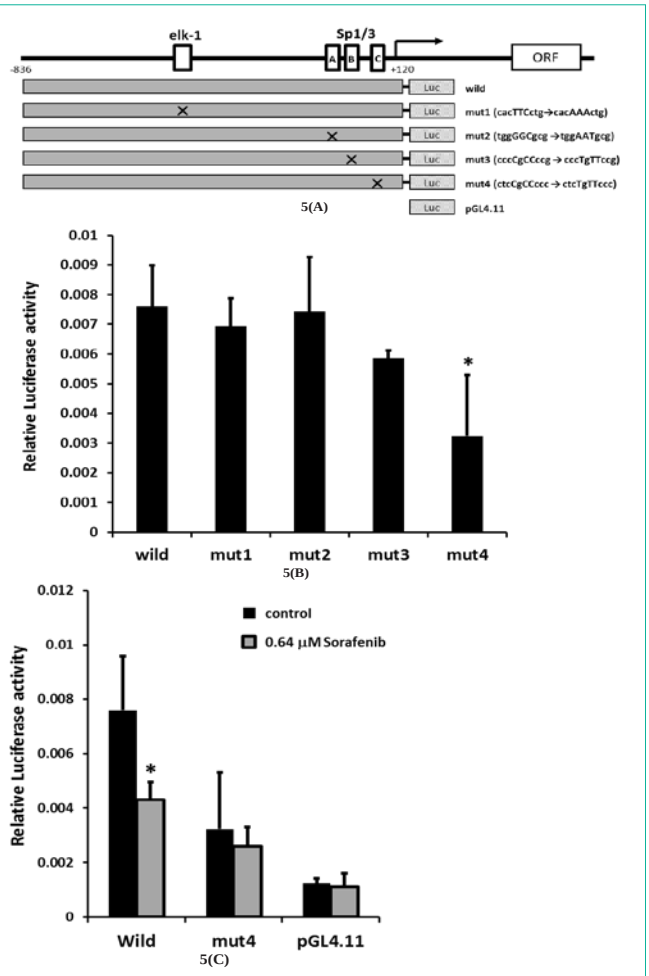

Figure 5: Site-directed mutagenesis of the DPYD promoter and activities of the mutated promoters. (A): Mutated constructs used in the reporter assay to determine the responsiveness of the DPYD promoter constructs. The X mark indicates the mutation sites on the DPYD promoter. The arrow shows the transcriptional start site of the DPYD gene. (B): Mutagenesis in the region -836 to +120. HepG2 cells were cultured for 16h after co-transfection with 2μg of the pGL 4.11/-836_+120 or its mutants and 0.1μg of pRL-SV40. Luciferase activities were determined by a dual-luciferase assay. The results obtained in three independent experiments are expressed as mean ± SD. Asterisks indicate significant differences (P< 0.05) as compared to the wild-type construct-transfected HepG2 cells. (C): Effect of sorafenib on Sp1/3C-mutated DPYD promoter construct in HepG2 cells. After 2μg of mut4 plasmid and 0.1μg of pRL-SV40 were co-transfected into HepG2 cells, these cells were incubated for 16h and then treated with 0.64μmol/L sorafenib. Promoter activities were obtained by dual-luciferase assay 16h post-treatment with sorafenib.

mRNA expression levels were determined by real-time PCR. As shown in Figure 4A, DPYD mRNA expression levels were suppressed by treating the cells with 6.25 and 12.5 μmol/L of U0126. Zhang et al. reported that the constitutive expression of DPYD was regulated by three Sp1/Sp3-binding sites (Sp1/3A, Sp1/3B and Sp1/3C) at -174 nt upstream of the transcriptional start site in HeLa cells [11]. In contrast, Raf-1, a target molecule of sorafenib, triggers the MEK/ERK/elk-1 signaling pathway. Thus, we determined whether an elk-1 binding site was present on DPYD using Match programs (http://www.gene-regulation.com/index.html) for -1000 nt upstream of the transcriptional start site of DPYD. The results revealed the presence of elk-1 consensus sites in the -523 to -517 nt region. The 5-flanking region of DPYD, -836 to +120 nt (-836_+120), was amplified from genomic DNA by PCR and cloned into the luciferase expression vector pGL4.11, resulting in the pGL4.11/-836_+120 plasmid. After transfection into HepG2 cells, HepG2 cells were treated with 0–12.5 μmol/L of U0126 for 16h, and luciferase activities were determined. As shown in Figure 4B, the promoter activity of pGL4.11/-836_+120, which contains one elk-1 and three Sp1/3 binding sites, was significantly reduced by U0126 treatment. These results indicated that DPYD gene expression and promoter activity were regulated by MEK/ERK signaling.

Site directed mutagenesis of DPYD

To determine whether Sp1/3A, B, C, and/or elk-1 are involved in the sorafenib-induced down-regulation of DPYD gene transcription, we prepared constructs in which the consensus sequence for elk-1, and Sp1/3A-C sites were mutated (Figure 5A).

The mutated plasmids were transfected into HepG2 cells and luciferase activities were determined. As shown in Figure 5B, the luciferase activity of mut4 (Sp1/3C-mutated construct) decreased compared to that of the wild type construct. These results suggest that the constitutive expression of DPYD does not depend on elk-1, Sp1/3A, Sp1/3B consensus sites in the 5′-flanking region of DPYD gene in HepG2 cells.

To investigate whether the Sp1/3C consensus sequence is necessary for sorafenib to decrease DPYD promoter activity, we determined the promoter activity of the DPYD mut4 construct. As shown in Figure 5C, DPYD promoter activity was suppressed by 0.64μmol/L of sorafenib, while that of DPYD mut4 was not suppressed. These result indicated that the Sp1/3C site on the DPYD promoter is essential for sorafenib to decrease DPYD mRNA expression levels.

DPYD has been thoroughly studied because it is the most functional enzyme involved in the inactivation of 5-FU during 5-FU chemotherapy. DPYD promoter was cloned in 2000 [15,16], and single nucleotide polymorphisms [17] and DNA methylation [18,19] in the promoter region have been reported. Transcriptional regulation of DPYD gene by Sp1 and Sp3 was previously reported [11]. The Sp family includes several members with a similar structure: Sp1–8 [20]. Sp1 and Sp3 are ubiquitously expressed in mammalian cells. Sp1 binds to the GC boxes located in the proximal promoters or enhancers found in almost all genes [21-23]. Xhang et al. claim that the Sp1/3B site is essential and that the Sp1/3C site works cooperatively with Sp1/3B, while Sp1/3A has minor promoter activity on DPYD in HeLa and HEK293 cells [11]. Our study indicates that Sp1/3C site is essential for the constitutive expression of DPYD in HepG2 cells. Although whether the functional sites are different remains an open question, we agree with Xhang et al. on that Sp1/3 is essential for the transcriptional regulation of DPYD in human cells. Sp1 interacts with some transcription factors and regulates their transcriptional activity. Lee et al. reported that MEK regulates molecular interactions between the inhibitory domain of Sp1 and co-repressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT), nuclear receptor co-repressor (NCoR), and BCL-6 interacting corepressor (BcoR) [24]. Especially, the interaction between SMART or NCoR and Sp1 is inhibited by MEK activation, resulting in an increase in the transcription activity via Sp1 binding to the GC boxes in the promoter or the enhancer. In contrast, MEK activation promotes the interaction between BcoR and Sp1. Thus, one could speculate that SMRT or NCoR may be released from Sp1 by MEK on the DPYD gene promoter. Considering our results, we speculate that sorafenib may enhance the sensitivity of HCC to HAIC-therapy using 5-FU by down-regulating DPYD expression via inhibition of the Raf/MEK/ERK cascade. Other reports indicated that sorafenib sensitizes cells to apoptosis [5], changes cytokine levels in sera [8], suppresses tumor vessel growth [4], and improves hypoxia conditions [9]. Sorafenib may increase the sensitivity to 5-FU in aHCC through these multiple effects as well. Further studies are warranted to investigate the novel mechanisms of action of sorafenib.

In this study, we clarified that sorafenib decreases DPYD expression by inhibiting the MEK pathway. Moreover, the Sp1/3C binding site on DPYD promoter is necessary for sorafenib to regulate DPYD expression. It is hoped that our study will contribute to a better understanding of chemotherapy using sorafenib and 5-FU against HCC.

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