Effect of 5-fluorouracil on mRNA expression of drug metabolizing enzyme and transporter genes in human hepatoma cell lines

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ABSTRACT

Fluoropyrimidines such as 5-fluorouracil (5-FU) are well known to have drug–drug interactions with anticoagulant medications such as warfarin. This study investigated the mRNA expression of pharmacokinetic (PK)-related genes in response to 5-FU using the hepatocarcinoma cell lines after examining relevant gene expression via RNA sequencing. We used HepaRG cells for 5-FU treatment analysis because these cells displayed PK-related gene expression. 5-FU exposure significantly reduced cytochrome P450 3A4 (CYP3A4) mRNA expression. Additionally, the mRNA expression of nuclear receptor subfamily 1 group I member 2 (also known as pregnane X receptor), a nuclear receptor transcription factor that promotes the expression of many CYP genes, was also decreased in HepaRG cells following 5-FU treatment. The mRNA expressions of the CYP2B6 and ATP-binding cassette transporter genes were decreased after 5-FU treatment. This study revealed that 5-FU treatment reduced PK-related gene expression in HepaRG cells. These findings should be useful for further drug–drug interaction research.

INTRODUCTION

Fluoropyrimidine anticancer agent such as 5-fluorouracil (5-FU) and its prodrugs are widely prescribed to treat several cancers including breast and colorectal cancers (Rich et al. 2004). After being metabolized, 5-FU enters a complex anabolic process that causes cytotoxicity at the cellular level by interfering with normal DNA and RNA function (Rich et al. 2004). Several mechanisms are responsible for this cytotoxic activity of 5-FU: 1) conversion of 5-FU to 5-fluoro-2-deoxyuridine monophosphate, an inhibitor of thymidylate synthase in a ternary complex with 5-methyltetrahydrofolate, which inhibits DNA synthesis (Pellino et al. 1985; Longley et al. 2003), 2) 5-FU causes DNA damage, precisely double-strand (and single-strand) breaks, during S phase because of the misincorporation of 5-fluorodeoxyuridine triphosphate into DNA (Curtin et al. 1991; Peters et al. 2000); and 3) disruption of normal RNA processing and function because of the incorporation of the 5-FU metabolite, fluorouridine triphosphate (FUTP), into RNA (Longley et al. 2003).

Several proteins families including enzymes and transporters are related to pharmacokinetics in humans. The human genome carries 57 cytochrome P450 (CYP) genes that are divided into 18 families. CYPs are among the most critical monooxygenases, and 15 CYP enzymes in families 1, 2, and 3 metabolize xenobiotics, including the majority of small-mol-

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ecule drugs currently in use (Guengerich et al. 2005). Inhibition of CYP enzymes is the most common cause of harmful drug–drug interactions that can lead to toxicity or a lack of efficacy through increased bioavailability or decreased elimination of compounds dependent on hepatic metabolism for systemic clearance. 5-FU is known to cause drug–drug interactions when used concurrently with other drugs metabolized by CYPs, resulting in elevated blood drug concentrations, but 5-FU does not directly inhibit CYP enzymes (Park et al. 2003). Some case reports detailed the interaction of 5-FU with warfarin, which is a substrate of CYP2C9 and CYP3A4 (Copur et al. 2001; Camidge et al. 2005; Isaacs et al. 2005; Saiif et al. 2005; Yamamuro et al. 2011). These reports discussed that inhibition of CYP2C9 by 5-FU increased the blood concentration of the warfarin optical isomer S-warfarin. 5-FU metabolites inhibit DNA synthesis and disrupt RNA function; therefore, 5-FU may disrupt CYP protein expression rather than directly inhibit CYP enzymes. However, the mechanism by which 5-FU inhibits CYPs is unclear. The ATP-binding cassette transporter gene ABCB1, which encodes p-glycoprotein, and ABCC2, which encodes multidrug resistance-associated protein 1, are related to severe adverse events induced by the docetaxel, cisplatin, and 5-FU combination chemotherapy (Nomura et al. 2019). These transporters are heavily involved in drug disposition (Alexander et al. 2019). Flavin-containing monooxygenases (FMOs) play a prominent role in phase I drug metabolism and constitute the second most important human monooxygenase system after CYPs (Cashman 2004). These proteins are closely related to pharmacokinetics in human.

Human hepatic cell lines are useful tools for evaluating drug metabolism. The human hepatocellular carcinoma cell line HepG2 has been widely used as a hepatocyte model for evaluating drug metabolism. The HepaRG cell line is a newly established hepatoma cell line obtained from a human hepatocellular carcinoma (Gerets et al. 2012; Yokoyama et al. 2018). To date, it is unclear whether 5-FU affects pharmacokinetic (PK)-related genes in human hepatocellular carcinoma cells. This study investigated whether 5-FU inhibits PK-related gene expression using the hepatocarcinoma cell lines.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO) and rifampicin (RIF, CYP3A4 inducer; positive control) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 5-FU was purchased from LKT Laboratories (St Paul, MN, USA). All reagents were biochemical grade.

Cell culture. HepG2 cells (European Collection of Authenticated Cell Cultures, Salisbury, UK) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Waltham, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) and Antibiotic-Antimycotic (100×) liquid (Thermo Fisher Scientific) at 37°C under 5% CO₂ and 95% air. HepaRG cells (KAC, Kyoto, Japan) were maintained in HepaRG Thawing and Seeding Medium 670 (KAC) and HepaRG Maintenance and Metabolism medium 620 (KAC) at 37°C under 5% CO₂ and 95% air according to the manufacturer’s instructions.

RNA extraction. HepG2 and HepaRG cells (5 × 10⁵ cells/well) were cultured in monolayers at 37°C for 24 h in 24-well collagen type I-coated plates (Iwaki Glass, Chiba, Japan). After preincubation for 24 h, the cells were collected, and total RNA was isolated using an RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). Total RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an RNA 6000 Nano Chip (Agilent Technologies) to determine the RNA integrity number (RIN) (Schroeder et al. 2006) and 28S:18S RNA values in all samples.

RNA-sequencing (RNA seq). RNA-seq was outsourced to Cancer Precision Medicine (Kanagawa, Japan). We obtained the FASTQ files with a read length of 2 × 150 bp. After filtering using Trimmomatic software (Bolger et al. 2014), the reads were analyzed using RSEM software (Li et al. 2011). Briefly, the sequencing reads were aligned to the human reference genome (GRCh37) using Bowtie 2 (Langmead et al. 2012). The relative abundance of each transcript in the annotation data for GRCh37 in the UCSC Genome Browser (Haussler et al. 2019) was estimated as Transcripts Per Million (TPM) (Wagner et al. 2012). The coverage of each isoform was calculated depending on the transcript. RNAseq was performed as a single experiment.

Cytotoxicity test. After preculture for 24 h, HepaRG cells were cultured in DMEM supplemented with RIF (10 μM) or 5-FU (0.1, 1, or 10 μg/mL) for 96 h. After culture, cells were immediately removed and then stored at −80°C until further analysis. These experiments were performed in biological quadrupli-
Table 1 Gene-specific primers for the qRT-PCR of 11 genes

| Gene symbol | Forward                     | Reverse                      |
|-------------|-----------------------------|------------------------------|
| ABCB1       | CAAAGAGGCTCTGGATGAAA        | AGCTTTGCCCAAATGTGAAAC        |
| ABCC2       | ATGGAAACAATTGTAGAAAGG       | GTTTCTCAAGGACCTTCAGATG       |
| ACTB        | AGCAAGAGATGGCCACAG          | TGACCTCTATTGTGCCTGGG         |
| CYP2B6      | CCCCATACCCCATCTCTTTT       | ACTTTGAGGCTGAGCTGGGA         |
| CYP2C9      | TTGTCCCTGCCCCGGAGATG       | CATGTTAGGGGCATGGG           |
| CYP2C19     | CAAACACCTTCGAGCTTTTA       | CATCCTGTTAGGGGCATGGG         |
| CYP2D6      | TGGTCTCCTGCCCCGGAGATG      | GTGATGAGTGGTGCCTCCCTTAG      |
| CYP3A4      | AGTGTTGGGGCTTTATGTG        | CATCCTCCTGAGTTTCCAC         |
| CYP3A5      | AGTGCAAGAACTCTGCAAGG       | TGCAGTTTCTCGTAGACATC         |
| FMO3        | CCCACCTGTTTGGAGAAAGG       | TCCGGAATCTGTGTTGTTG          |
| NR1I2       | CAGGAGCAATTTCGCCATT         | GGTGAGCATAGCCATGATC          |

ABCB1, ATP binding cassette subfamily B member 1; ABCC2, ATP binding cassette subfamily C member 2; ACTB, β-actin; CYP, cytochrome P450; FMO3, flavin-containing monooxygenase 3; NR1I2, nuclear receptor subfamily 1 group 1 member 2.

cates. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) to assess gene expression was also performed in quadruplicate.

qRT-PCR. After treatment for 96 h, total RNA was isolated as described previously. Total RNA (0.5 μg) was used to synthesize cDNA using PrimeScript RT Master Mix (TAKARA, Shiga, Japan) according to the manufacturer’s protocol. cDNA was amplified using TB Green Premix Ex Taq II (TAKARA) and gene-specific primers (Table 1) in a 7500 HT Fast Real-time PCR System (Thermo Fisher Scientific). The PCR conditions were as follows: 95°C for 10 sec; 40 cycles of 95°C for 5 sec, 60°C for 30 sec; 95°C for 15 sec and 60°C for 10 min. The cycle threshold (Ct) values were obtained, and all mRNA level values were normalized to those of β-actin (ACTB). Relative expression levels compared with DMSO-treated samples are presented as fold reduced calculated by the 2^−ΔΔCt method. qRT-PCR was performed in biological quadruplicates.

Statistical analysis. All values were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Relative expression levels are presented as the mean ± SEM (n = 4). Statistical differences among treatments were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s test. The level of significance was taken as P < 0.05.

RESULTS

Gene expression analysis in HepaRG and HepG2 cells

The RNA sequencing results for HepG2 and HepaRG cells are presented in Table 2. Finally, 32,803 genes and 82,442 transcripts were determined. The TPM for each PK-related gene, including CYP2C9, CYP2C19, and CYP3A4, were elevated in HepaRG cells but not in HepG2 cells (Table 2). Therefore, the effects of 5-FU on these genes were evaluated using the HepaRG cell line.

Effect of 5-FU on PK-related mRNA expression in HepaRG cells

The effects of RIF or 5-FU exposure to PK-related mRNA expression are shown in Figure 1. CYP3A4 mRNA expression was significantly increased to 240.32% compared with its expression in DMSO-treated control cells after exposure to 10 μg/mL RIF for 96 h. Therefore, it was confirmed that HepaRG cells can be used to evaluate PK-related gene expression. After exposure to 10 μg/mL 5-FU for 96 h, CYP3A4 mRNA levels were significantly reduced to 21.87% compared with that in DMSO-treated control cells. The mRNA expression of CYP2C9, CYP2C19, and CYP3A4 was also significantly decreased to 31.41%, 53.49%, and 42.03%, respectively, compared with that in DMSO-treated control cells. CYP2C9 mRNA expression was not altered by 5-FU treatment. The mRNA expression of nuclear receptor subfamily 1 group 1 member 2 (NR1I2, also known as pregnane X receptor [PXR]) was significantly reduced to 24.35% compared with that in DMSO-treated control cells. Exposure to 0.1 or
RIF-induced CYP3A4 mRNA expression, as a positive control, was observed only in HepaRG cells. RIF is known to more strongly induce the expression of CYP3A4 than other CYPs, phase II enzymes, and transporters (Rae et al. 2001). HepG2 cells have been revealed to have low level of CYP function (Gerets et al. 2012). HepaRG cells were developed to overcome the low metabolic activity of HepG2 cells, and former cell line displays similar metabolic function and liver-specific gene expression as of primary human hepatocytes (Aninat et al. 2006; Gerets et al. 2012). Our findings agree with these reports. We found that NR1I2, CYP3A4, CYP2B6, ABCB1, and FMO3 mRNA levels were significantly decreased in HepaRG cells by 96 h treatment with 10 μg/mL 5-FU. Notably, reduction in the mRNA expression of NR1I2, which plays an important role in CYP gene expression, was observed. Aryl hydrocarbon receptor (AhR), NR1I2, and constitutively 1 μg/mL 5-FU for 96 h did not significantly alter CYP mRNAs expression, but NR1I2 and ABCB1 expression was significantly reduced by exposure to 1 μg/mL 5-FU for 96 h to 61.73% and 70.06% respectively, compared with that in DMSO-treated control cells. Exposure to 0.1 μg/mL 5-FU for 96 h did not significantly affect PK-related mRNA expression. 5-FU treatment downregulated several PK-related genes, including CYPs, transporters and FMO3.

DISCUSSION

In the present study, we investigated genome-wide PK-related gene expression variability using RNA sequencing in HepG2 and HepaRG cells, and then examined whether 5-FU treatment affects PK-related gene expression. The expression of 10 PK-related genes was confirmed in HepaRG cells. Additionally, Table 2

| Gene symbol | Transcript ID | HepaRG | Percentage of isoform TPM | HepG2 | Percentage of isoform TPM |
|-------------|---------------|--------|---------------------------|-------|---------------------------|
| ABCB1       | NM_000927.4   | 1.04   | 3.25                      | 2.03  | 4.15                      |
| ABCB1       | NM_001348944.1| 0      | 0                         | 0     | 0                         |
| ABCB1       | NM_001348945.1| 0      | 0                         | 0     | 0                         |
| ABCB1       | NM_001348946.2| 30.79  | 96.75                     | 46.97 | 95.85                     |
| ABCC2       | NM_000392.5   | 110.34 | 100                       | 112.66| 100                       |
| CYP2B6      | NM_000767.5   | 2.7    | 100                       | 0.08  | 100                       |
| CYP2C9      | NM_000769.4   | 1.16   | 100                       | 0     | 0                         |
| CYP2C19     | NM_000771.4   | 5.81   | 100                       | 0.09  | 100                       |
| CYP2D6      | NM_001010.6   | 0      | 0                         | 0.59  | 30.72                     |
| CYP2D6      | NM_001025161.3| 0.62   | 100                       | 1.33  | 69.28                     |
| CYP2D6      | NM_001010.6   | 0      | 0                         | 0     | 0                         |
| CYP3A4      | NM_001202855.3| 10.12  | 4.79                      | 0     | 0                         |
| CYP3A4      | NM_017460.6   | 201.35 | 95.21                     | 0.55  | 100                       |
| CYP3A5      | NM_000777.5   | 11.29  | 32.64                     | 4.92  | 83.96                     |
| CYP3A5      | NM_01190484.3 | 6.19   | 17.91                     | 0.34  | 5.72                      |
| CYP3A5      | NM_001291829.2| 4.72   | 13.64                     | 0.42  | 7.11                      |
| CYP3A5      | NM_001291830.2| 4.71   | 13.62                     | 0     | 0                         |
| CYP3A5      | NR_023807.3   | 7.68   | 22.19                     | 0.19  | 3.2                       |
| FMO3        | NM_00102294.3 | 43.22  | 85.75                     | 0     | 0                         |
| FMO3        | NM_001319173.2| 1.53   | 3.03                      | 0     | 0                         |
| FMO3        | NM_001319174.2| 0      | 0                         | 0     | 0                         |
| FMO3        | NM_006896.4   | 5.65   | 11.22                     | 0     | 0                         |
| NR1I2       | NM_003889.3   | 1.91   | 22.78                     | 1.35  | 17.49                     |
| NR1I2       | NM_022002.2   | 6.49   | 77.22                     | 5.72  | 74.23                     |
| NR1I2       | NM_033013.2   | 0      | 0                         | 0.64  | 8.28                      |

ABCB1, ATP binding cassette subfamily B member 1; ABCC2, ATP binding cassette subfamily C member 2; CYP, cytochrome P450; FMO3, flavin-containing monooxygenase 3; NR1I2, nuclear receptor subfamily 1 group I member 2; TPM, transcripts per million.
5-FU exposure significantly reduced NR1I2 and ABCB1 mRNA expression in HepaRG cells. CYP3A4 mRNA expression was decreased only by 10 μg/mL 5-FU exposure. In line with previous findings that NR1I2 regulated CYPs and ABCB1 mRNA expression, the mechanism of our results may be considered that the reduction of CYPs and ABCB1 mRNA expression was initiated by a decrease in the mRNA expression of NR1I2, which regulates each gene.

CYP inhibition can be irreversible (mechanism-based inhibition) or reversible. Moreover, mechanism-based inhibition is terminated by enzyme re-synthesis and is usually long-lasting (Pelkonen et al. 2008). The decreased levels of CYPs mRNA expression by 5-FU may be considered the latter. Therefore, 5-FU mediated drug–drug interaction effect may persist for a long period compared with re-
versible inhibition such as directly binding CYP catalytic inhibition.

CYP2C9 has been strongly suspected to participate in drug–drug interaction between warfarin and 5-FU (Copur et al. 2001; Camidge et al. 2005; Isaacs et al. 2005; Saif et al. 2005; Yamamura et al. 2011). However, our present data did not indicate a significant fluctuation of CYP2C9 mRNA expression. PXR, CAR, and the glucocorticoid receptor (GR) regulate CYP2C9 gene expression and hepatocyte nuclear factor 4α (HNF4α), and these factors are closely connected (Urquhart et al. 2007; Pelkonen et al. 2008). In addition to NR112, CAR, GR, HNF4α, farnesoid X receptor, and vitamin D receptors participate as nuclear receptors that regulate drug-metabolizing enzymes and transporters (Urquhart et al. 2007; Lin et al. 2008; Rigalli et al. 2013). From these reports, the involvement of nuclear receptors may be critical for CYP2C9 gene expression following 5-FU treatment responsibility. Because 5-FU treatment suppressed CYP3A4 mRNA but did not CYP2C9 mRNA expression, CYP3A4-mediated drug metabolism might be partially involved in 5-FU-induced drug–drug interaction mechanism. Although significantly decreased FMO3 mRNA expression was observed, the responsible mechanism is unclear.

This study had a few limitations. First, our experiments only investigated mRNA expression in hepatocarcinoma cells. Protein expression experiments are needed to determine the actual expression of CYPs in HepaRG cells and other cells such as primary hepatocytes. Second, we used a 96 h incubation period for 5-FU treatment. Our presented data revealed no significant decreases in CYP2C9 mRNA expression after exposure to 5-FU. The effect of the exposure time on mRNA fluctuations remains unresolved. A previous mRNA expression study treated cells with 5-FU for 24 h (Rigalli et al. 2013). In contrast, clinical reports suggested that at least a few weeks are needed to assess between 5-FU and warfarin to clarify the suspected involvement of CYP2C9 inhibition in increasing the international normalized ratio (Copur et al. 2001; Park et al. 2003; Camidge et al. 2005; Isaacs et al. 2005; Saif et al. 2005). Further experiments are required to investigate the time-course effects of 5-FU treatment.

In conclusion, we evaluated the effect of 5-FU on the mRNA expression of PK-related genes in human adenocarcinoma cell lines. 5-FU treatment reduced NR112, CYP2B6, CYP3A4, and ABCB1 mRNA expressions in HepaRG cells. These findings are considered useful information for drug–drug interaction research.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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