Tivantinib Decreases Hepatocyte Growth Factor-Induced BCRP Expression in Hepatocellular Carcinoma HepG2 Cells

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary liver cancer derived from hepatocytes and accounts for approximately 90% of all cases of primary liver cancer. Currently, sorafenib (first-line),1 regorafenib (second-line),2 and lenvatinib (first-line)3 are used as molecular target therapeutic agents for HCC. In recent years, ramucirumab, tivantinib, and others have been evaluated as new chemotherapy agents and clinical trials are underway.4–6

Mesenchymal–epithelial transition factor (cMET), a selective receptor for hepatocyte growth factor (HGF), is a receptor tyrosine kinase oncogene.7 cMET is highly expressed in many types of malignant tumors, epithelial cells, and non-epithelial cells.8 Thus, many inhibitors of cMET have been developed.7 Tivantinib is an ATP non-competitive selective inhibitor of cMET and is believed to exert antitumor effects by inhibiting intracellular signal transduction mediated by HGF, which transduces RAS/RAF/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/FAK, AKT/mammalian target of rapamycin (mTOR), and CDC42/RAC1 signals. Tivantinib has shown promising effects against HCC in Phase II trials, with positive results in patients with high expression of cMET.9 However, the results of Phase III trials did not reveal a significant difference in overall survival as compared to the placebo group.9 Additionally, few studies have evaluated sensitization to hepatic arterial infusion chemotherapy (HAIC) with 5-fluorouracil (FU), via tivantinib pretreatment in HCC.

Previously, we reported that sorafenib influences 5-FU metabolism in HCC, and that the antitumor effect of HAIC with 5-FU is enhanced by pre-administration of sorafenib.10 This finding suggests that pre-administration of molecular targeting drugs before HAIC significantly extends the survival of patients with HCC.

Various other factors also impact the metabolism of chemotherapeutic drugs, including proteins and enzymes. For instance, dihydropyridine dehydrogenase (DPYD) is the most functional enzyme associated with inactivating 5-FU in chemotherapy. DPYD catalyzes the rate-limiting step of 5-FU metabolism. Therefore, upregulation of its activity serves to decrease the cytotoxicity of 5-FU in cancerous cells.11 Similarly, breast cancer therapy-resistant protein (BCRP) is a member of the ATP-binding cassette (ABC) family and is among the major transporters that excrete drugs, such as sorafenib and 5-FU, from the intracellular environment into the bile.12 The expression of BCRP also affects the efficacy of 5-FU.

In this study, we investigated the effects of tivantinib on cMet-mediated 5-FU metabolism and transport by focusing on the expression of BCRP and DPYD in HCC cell lines.

Note

Tivantinib, a mesenchymal–epithelial transition factor (cMET) inhibitor, is a molecular targeting drug that kills hepatocellular carcinoma (HCC) cells. Tivantinib alone does not affect the overall survival of patients with HCC, and combination treatment with tivantinib and other therapies has not been evaluated. This study was conducted to clarify the effect of the tivantinib in regulating breast cancer therapy-resistant protein (BCRP), a key transporter of 5-fluorouracil (5-FU), and dihydropyridine dehydrogenase (DPYD), a major metabolic enzyme of 5-FU. To this end, cMET gene expression was determined by RT-PCR in HepG2 (human hepatoma) cells. The transcriptional start sites of BCRP were determined by 5′-rapid amplification of cDNA ends (5′-RACE). BCRP and DPYD mRNA levels were determined by real-time RT-PCR, and promoter activities were measured by dual-luciferase assays. Results show that hepatocyte growth factor (HGF) upregulated the mRNA level of BCRP, but not DPYD, in HepG2 cells. The upregulation of BCRP expression by HGF was down-regulated by tivantinib. We also identified two transcriptional start sites (E1a, E1β) in BCRP by 5′-RACE. The transcriptional activity of the region −287 to E1a of BCRP was upregulated by HGF, which was decreased by tivantinib, whereas activity of the region −297 to E1β of BCRP was not affected by tivantinib. Therefore, tivantinib regulates BCRP expression upstream of exon 1a. Combination treatment of tivantinib and 5-FU should be further evaluated for HCC therapy.

Key words tivantinib; hepatocellular carcinoma; 5-fluorouracil; breast cancer therapy-resistant gene

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**MATERIALS AND METHODS**

**Cell Culture** HepG2 (human hepatoma) cells and HuH7 (well-differentiated human hepatocellular carcinoma) cells were purchased from the Japanese Cancer Research Resources Cell Bank (Tokyo, Japan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; WAKO, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS, U.S.A.).

**Western Blotting for cMet and Phosphorylated cMet** Cells were collected and resuspended in 500 µL of 1× radio immunoprecipitation assay (RIPA) lysis buffer containing Complete, ethylenediaminetetraacetic acid (EDTA)-free (Sigma-Aldrich, St. Louis, MO, U.S.A.) and disrupted by sonication thrice, for 10 s intervals on ice with a Model VP-5S Ultra Homogenizer (Taitec Co., Tokyo, Japan). The lysate was then centrifuged at 15000 rpm for 15 min and the supernatant was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a Hybond-P polyvinylidene fluoride membrane (GE Healthcare, Uppsala, Sweden), the following primary antibodies were used: 0.5 µg/mL anti-cMet rabbit antibody (Proteintech, Rosemont, IL, U.S.A.) and 2 µg/mL anti-phosphorylated cMET rabbit antibody (Rockland Immunochemicals, Limerick, PA, U.S.A.) in Can Get Signal Solution-I (Toyobo, Tokyo, Japan), followed by further incubation with 0.025 µg/mL peroxidase (POD)-labeled anti-rabbit immunoglobulin G (IgG) (H + L) secondary antibody (Bethyl Laboratories, Montgomery, TX, U.S.A.) in Can Get Signal Solution-II (Toyobo). The POD-labeled anti-β-actin antibody (1 : 20000; Cosmo bio, Tokyo, Japan) in Can Get Signal Solution-II was used as an internal control. The membrane was detected with Luminata Crescendo (Millipore, Billerica, MA, U.S.A.) followed by analysis with a LuminoGraph (ATTO, Tokyo, Japan).

**RT-PCR to Evaluate cMet Expression** Total RNA was purified from HepG2 cells using RNAiso Plus (TaKaRa Bio, Otsu, Japan) according to the manufacturer’s instructions. cDNA was generated from total RNA using ReverTra Ace reverse transcriptase (Toyobo) and an oligo (dT) 20 primers. cDNA was amplified in a PC-812 thermal cycler (Astec, Otsu, Japan) according to the manufacturer’s instructions. The PCR conditions for cMET were as follows: 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s. The PCR products were then amplified as described above using S2 and A2 primers.

**Real-Time RT-PCR for BCRP and DPYD** HepG2 cells (2 × 10^5 cells/mL) were seeded into 6-well plates and incubated for 16 h. After treatment with tivantinib (0–1 µg/mL; ChemScene, Monmouth Junction, NJ, U.S.A.) for 1 h, recombinant human (rh) HGF (Wako) was added and incubated for an additional 6 h. Total RNA was then extracted from the cells, cDNA was generated from total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo) according to the manufacturer’s instructions. The levels of BCRP and GAPDH mRNA were determined using specific primers (Table 1) with an ABI PRISM 7500 Fast (Life Technologies, Carlsbad, CA, U.S.A.) using THUNDERBIRD Sybr qPCR Mix (Toyobo) according to the manufacturer’s instructions. PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Data were analyzed and normalized to GAPDH using external-standard methods with GAPDH gene cloned into pGL4.11 vector.

**5'-Rapid Amplification of cDNA Ends (5'-RACE)** The 5’ end of BCRP cDNA was observed using a 5'-Full RACE Core Set (TaKaRa) according to the manufacturer’s instructions. First-strand cDNA was synthesized from total RNA obtained from HepG2 cells using RT-Primers. The reaction mixture was then digested with ribonuclease (RNase) H and ligated with T4 ligase. The ligated DNA was used as a template for the first PCR amplification, using S1 and A1 primers. PCR conditions were 94°C for 3 min followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s. The PCR products were then amplified as described above using S2 and A2 primers. A sample of the PCR product was electrophoresed on a 2% agarose gel and visualized with ethidium bromide. The remaining PCR mixture was ligated into the EcoRV-digested pGL4.11 vector (Life Technologies) using an In-Fusion HD cloning kit (TaKaRa) according to the manufacturer’s instructions, followed by sequencing on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

**Reporter Construction for Luciferase Assay** The −287_Ela and −297_E1β sequences upstream of exon1 in BCRP were amplified by PCR from HepG2-derived genomic DNA using PrimStarMAX (TaKaRa) and specific primers: −287_Ela forward: 5'-GCC TCG CGG GCC AAC GCT TT GG-3' and reverse: 5'-TGG CCG GAG GCC AGC GA-3'; −297_E1β forward: 5'-CGG CTT AGG AAG TTC GTG TC-3' and reverse: 5'-CCT TCC CCC AACC ACC ACC-3'. The PCR products were phosphorylated by T4 polynucleotide kinase (Toyobo) and ligated into the pGL4.11 vector.

**Dual-Luciferase Reporter Gene Assay** For this dual-
luciferase assay, 2 μg of −287_E1α and −297_E1β reporter plasmids with 0.1 μg of pRL-SV40 (Promega, Madison, WI, U.S.A.) were co-transfected into HepG2 cells (20 × 10^4 cells) using Polyethylenimine “Max” (Polysciences, Warrington, PA, U.S.A.).

The co-transfected cells were exposed to 40 ng/mL of HGF and 0–1.0 μmol/L of tivantinib for 24 h, after which luciferase activities were measured with a Dual-Luciferase Assay Kit (Promega), according to manufacturer’s instructions, and 20/20n luminometer (Promega). The luciferase activity of each reporter plasmid was normalized to SV40-Luc activity in transfected cells.

**Statistical Analysis** All experimental data are presented as mean ± standard deviation (S.D.) and were analyzed by Student’s t-tests. Statistical significance was defined as p < 0.05.

**RESULTS AND DISCUSSION**

**cMET mRNA and Protein Expression in Huh7 and HepG2 Cells** HepG2 and Huh7 cell lines were used. To clarify the regulation of BCRP and DPYD expression by tivantinib in HCC cells, we first determined the mRNA and protein expression of cMET by RT-PCR (Fig. 1) and Western blotting (data not shown), respectively.

Compared to Huh7 cells, HepG2 cells showed higher expression of cMET protein and mRNA. Therefore, subsequent experiments were carried out using HepG2 cells stimulated with HGF.

**Changes in mRNA Levels of BCRP and DPYD in HepG2-Stimulated with HGF in the Presence of Tivantinib** To investigate whether the mRNA expression levels of BCRP and DPYD are altered by HGF stimulation in the presence of tivantinib, we determined the transcript levels of these genes. Figure 2A shows that BCRP mRNA in HepG2 cells was upregulated by HGF. However, this effect was suppressed by pretreatment with tivantinib. In contrast, DPYD mRNA expression was not affected by HGF stimulation or tivantinib pretreatment in HepG2 cells (Fig. 2B). These results demonstrate that tivantinib affects BCRP regulation by HGF stimulation via cMET signaling.

**Transcriptional Start Site and 5′-Flanking Region of BCRP in HepG2 Cells**

The BCRP gene (NT_022959), ATP-binding cassette super-family G member 2 (ABCG2), is composed of 16 exons on chromosome 4 (4q22). Although many alternative promoters of ABCG2/BCRP have been reported, few studies have attempted to regulate the transcription of this gene in HepG2 cells.

To clarify the expression of ABCG2/BCRP in HepG2 cells, we determined its transcriptional start site by 5′-RACE, which revealed a region of approximately 200 base pairs, as shown in Fig. 3A. The transcriptional start sites of BCRP genomic DNA are shown in Fig. 3B. 5′-RACE analysis revealed exon 1 and transcriptional start sites of ABCG2/BCRP in HepG2 cells, express two transcripts of this gene.

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**Fig. 2. Changes in mRNA Levels of BCRP and DPYD in HepG2-Stimulated with HGF in the Presence of Tivantinib**

The BCRP (A) and DPYD (B) genes were amplified by real-time RT-PCR using gene-specific primers. The results were obtained from three to five independent experiments and the data represent the mean ± S.D. Asterisks indicate significant differences (p < 0.05).

**Fig. 3. Transcriptional Start Site and 5′-Flanking Region of BCRP Gene in HepG2 Cells**

(A) Results of 5′-RACE of BCRP mRNA in HepG2 cells. PCR products obtained from 5′-RACE were electrophoresed on 2% agarose gels and stained with ethidium bromide. (B) Position of transcriptional start site of BCRP in HepG2 cells.
BCRP Promoter Activity Induced Stimulation with HGF in the Presence of Tivantinib in HepG2 Cells

To understand the transcriptional regulation of the BCRP gene in HepG2 cells stimulated with HGF and the suppressive effect of pretreatment with tivantinib, we prepared \(-287\_E1\alpha\) and \(-297\_E1\beta\) constructs based on pGL4.11 (Fig. 3B). Figure 4A shows the changes in the transcriptional activity of the two regions containing the promoters of BCRP following HGF stimulation. Although the promoter activity of \(-287\_E1\alpha\) was upregulated, \(-297\_E1\beta\) was not changed by HGF stimulation.

Furthermore, the increased in transcriptional activity of \(-287\_E1\alpha\) stimulated with HGF was dose-dependently suppressed by pretreatment with tivantinib (Fig. 4B).

Many studies have suggested that HGF and cMET expression induce malignant transformation and metastasis.\(^\text{15}\) High expression of HGF/cMET may induce gene expression of BCRP, resulting in resistance to 5-FU and sorafenib and increased BCRP substrates.\(^\text{16}\) Based on our results, tivantinib can be applied in combination therapy with 5-FU as HAIC and sorafenib.

Numerous studies have evaluated the function and effect of tivantinib and have reported that it affects the expression of apoptosis-related molecules, while enhancing the antitumor effect of sorafenib in HCC by regulating sorafenib-related metabolism and transporter gene expression.\(^\text{17}\) Katayama et al. reported that tivantinib inhibits microtubule polymerization and cMET.\(^\text{18}\) This suggests that pre-administration of tivantinib before other antitumor drugs can increase the cytotoxicity of treatments for HCC.

In conclusion, our results may contribute to improvements in chemotherapy using tivantinib and 5-FU against HCC.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


