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Construction of HEK293 cells stably expressing wild-type organic anion transporting polypeptide 1B1 (OATP1B1*1a) and variant OATP1B1*1b and OATP1B1*15

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A transgenic cell line stably expressing the human organic anion transporting polypeptide (OATP1B1) was established. Human Embryonic Kidney 293 (HEK293) cell line stably expressing OATP1B1*1a sequence was amplified through PCR with the extracted total RNA as templates from human liver, then subcloned into the plasmid pMD19-T and verified by sequencing. OATP1B1*1b/OATP1B1*15 mutant sequences were obtained by site-directed mutation PCR with pMD19-T/ OATP1B1*1a as templates. The plasmids pcDNA3.1(+)/OATP1B1*1a, *1b and *15 were constructed and transfected into HEK293 cell line using Lipofectamine™2000 transfection reagent. Several stable transfected clones were obtained after selection with G418. Using rosuvastatin as a probe substrate of OATP1B1, the intracellular rosuvastatin accumulation in HEK293 and HEK-OATP1B1*1a, *1b and *15 monoclone cells were validated by a ultra-performance liquid chromatography-tandem mass spectrometry. OATP1B1 mRNA and protein expression were detected by RT-PCR and Western blot, respectively. The results from RT-PCR, rosuvastatin uptake and Western blot assay indicated that human OATP1B1 was highly expressed in transfected cells compared with controls. The HEK-293 cell lines stably expressing human OATP1B1-wild and variant (HEK-OATP1B1, *1b and *15) are potential models to study drug transport *in vitro*.

1. Introduction

During recent years, the plasma membrane influx and efflux transporters have increasingly been recognized as the key determinants in the rate and extent of drug intestinal absorption, tissue distribution as well as hepatic and renal clearance. Members of the organic anion-transporting polypeptide (OATP) family are drug uptake transporters mediating the active cellular influx of a variety of amphipathic compounds (Maeda 2015; Nakanishi and Tamai 2012; Zhou et al. 2014). OATP1B1 (encoded by the SLCO1B1 gene) is predominantly expressed at the basolateral membrane of human hepatocytes and is responsible for uptaking its substrates from blood into the liver (Konig et al. 2012; Hong et al. 2015). Due to its liver-specific tissue distribution characteristics and capacity of transporting a large number of structurally divergent compounds, OATP1B1 plays an important role in the hepatocellular uptake of endogenous compounds and xenobiotics (e.g., bilirubin and its glucuronide, bile acids, estrone sulfate, estradiol-17β-D-glucuronide, fexofenadine and several statins) (Zhou et al. 2013a, b; Niemi 2007).

Several sequence variations or single nucleotide polymorphisms (SNPs) have been reported in the SLCO1B1 gene (Niemi et al. 2011), some mutations are associated with altered transport activity of OATP1B1 in *vitro* and in *vivo* (Kameyama et al. 2005; Daka et al. 2015). SLCO1B1*1b (c.388G-c.521T) and SLCO1B1*15 (c.388G-c.521C) are common haplotypes in the Chinese population with frequencies of 59.9% and 14.0%, respectively (Xu et al. 2007). The SLCO1B1*1b/*1b genotype is associated with reduced lovastatin acid concentrations due to enhanced hepatic uptake (Tornio et al. 2015), whereas pitavastatin exposure was significantly increased in the SLCO1B1*15/*15 subjects versus SLCO1B1*1a/*1b subjects due to reduced hepatic absorption (Choi et al. 2012). Considering the important role of OATP1B1 and relative high variant allele frequency of SLCO1B1*1b and SLCO1B1*15, this study was initiated to construct the human embryonic kidney 293 (HEK-293) cell line stably expressing human OATP1B1*1a (c.388A/c.521T), OATP1B1*1b and OATP1B1*15 for studying the OATP1B1-mediated drug uptake *in vitro*.

2. Investigations, results and discussion

2.1. RT-PCR analysis of OATP1B1 mRNA expression

HEK-293 cells were stably transfected with pcDNA3.1/(+)-OATP1B1*1a, *1b and *15. G418-resistant monoclones and HEK-Control cell were screened for OATP1B1 expression by RT-PCR. As shown in Fig. 1, only 309-bp OATP1B1 cDNA fragment and 550-bp β-actin cDNA fragment were detected in HEK-OATP1B1 cells. In HEK-Control cells, no OATP1B1 was detected. That indicated the pcDNA3.1/(+)-OATP1B1*1a, *1b and *15 have been transfected and transcribed into the HEK-293 cells.

2.2. Uptake transport assays

The activity of HEK-OATP1B1 and HEK-Control cells were measured by the uptake of rosuvastatin. Significantly higher values were observed with the uptake of rosuvastatin into the №.5 HEK-OATP1B1*1a monoclone, the №.2 HEK-OATP1B1*1b monoclone and the №.3 HEK-OATP1B1*15 monoclone compared to HEK-Control cells (Fig. 2).

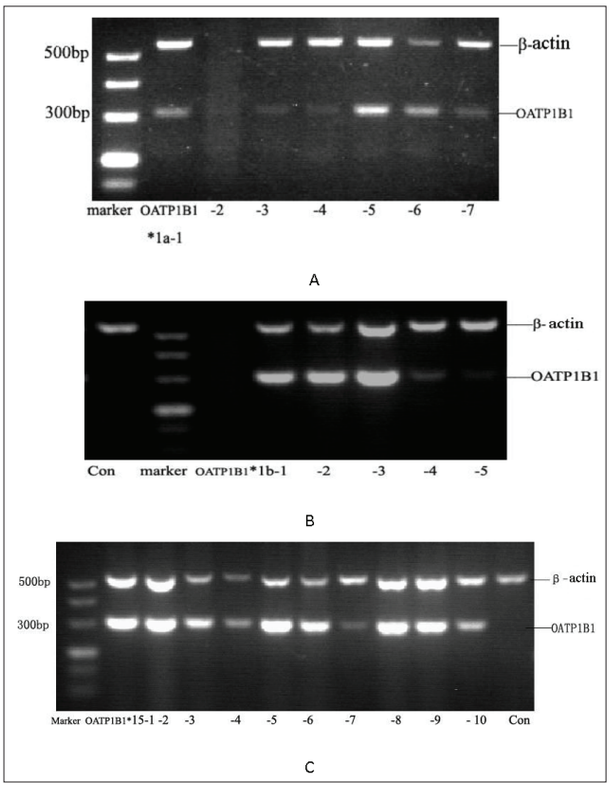

Fig. 1: OATP1B1 mRNA expression of HEK-con and HEK-OATTP1B1 monoclonal cells.
(A) HEK-OATTP1B1*1a; B) HEK-OATTP1B1*1b; (C) HEK-OATTP1B1*15

showed an apparent molecular mass of about 90 kDa in HEK-OATP1B1*1a, *1b and *15 monoclones.

3. Discussion

In this article, the results from RT-PCR, the intracellular rosuvastatin uptake and Western blot assay indicated that HEK293 cell lines stably expressing human OATP1B1-wild /mutants (HEK-OATP1B1*1a/*1b/*15) have been successfully established. Genetic polymorphism in OATP1B1 may have a great impact on the elimination of many drugs in clinical practice. The frequencies of the functionally significant variants of SLCO1B1 (i.e., SLCO1B1*1b and *15 haplotypes) were as follows: 59.9% and 14.0%, respectively, for Chinese; 46.9% and 3.7%, respectively, for Japanese; 26% and 16%, respectively, for Europe; 39% and 24%, respectively, for America (Pasanen et al. 2008). Therefore, the transgenic cells lines we established can be applied to study the OATP1B1*1a, *1b, *15 -mediated drug transport .

So far, several types of transgenic cells lines expressing OATP1B1 have been reported. AstraZeneca utilized in-house HEK293 cell cultures genetically transfected with OATP1B1 using probe substrate [^{3}H]-oestradiol 17beta-glucuronide) to identify substrates and inhibitors in drug development (Sharma et al. 2010), and demonstrated no indication of transporter-mediated uptake of montelukast in OATP2B1 and OATP1B1 expressing HEK293 cells (Brännström et al. 2015). Rosuvastatin is a widely prescribed HMG-CoA reductase inhibitor and is easily available. Also, OATP1B1 contributes predominantly to the hepatic uptake of rosuvastatin (Kitamura et al. 2008), therefore we used rosuvastatin as probe substrate of OATP1B1 in uptake transporter assay. Zimmerman et al. (2013) generated stable, isogenic Flp-In T-Rex293 cells expressing OATP1B1*1a, OATP1B1*5 or OATP1B1*15 to study transport of tyrosine kinase inhibitors. Lancaster et al. (2012) used stably transfected Flp-In T-Rex293 cells and proved erythromycin as a substrate for OATP1B1*1a with a Michaelis constant of ~13 μM. Pu et al. (2015) used transgenic human

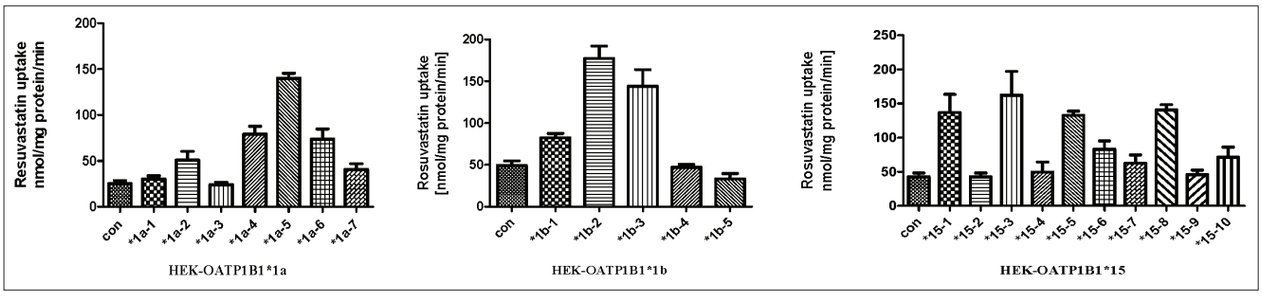

Fig. 2: Uptake of rosuvastatin into HEK-control and HEK-OATP1B1 monoclonal cells
Note: Data were expressed as mean±SD of three independent experiments.

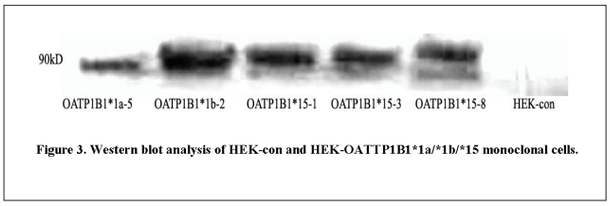

Fig. 3: Western blot analysis of HEK-con and HEK-OATTP1B1*1a/*1b/*15 monoclonal cells.

2.3. Western blotting assay

Expression of OATP1B1 in HEK-OATP1B1 cell lines was further confirmed by Western blot assay. As shown in Fig. 3, OATP1B1

breast cancer cells (MCF-7) expressing OATP1B1 wild-type 388GG and 521CC to determine whether the gene polymorphisms affected the therapeutic efficacy of tamoxifen for MCF-7.

Ni et al. (2010) evaluated the transport of flavopiridol and its glucuronide metabolite in uptake assays in HEK-293 and Madin-Darby canine kidney (MDCK-II) cells transiently transfected with SLCO1B1, and observed that transport may be affected by the different membrane and transporter compositions in the two cell lines because flavopiridol transport rates in HEK-293 cells were approximately 2-3 fold higher than in MDCK-II cells whereas flavo-glucuronide transport rates were similar in both cell lines (Ni et al. 2010). The transporting activities of OATP1B1*5 -expressing transgenic HeLa cells were reduced to less than half of those of OATP1B1*1a, whereas the activities of OATP1B1*5 -expressing transgenic HEK293 cells were similar with those of OATP1B1*1a (Kameyama et al. 2005). Taken together, head-to head comparison of transport efficiency in different transgenic cells need to be conducted in the future.

4. Experimental

4.1. Materials

Geneticin (G418) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco (Grand Island, NY, USA). OATP1B1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA,USA). Goat anti rat IgG(H+L) was purchased from Muti-Sciences Biotech (Hangzhou, China). HEK-293 cells were kept at our lab. ECL™ Western Blotting Detection Reagents was purchased from Biological Industries (BIOIND, Israel). pcDNA3.1(+), Lipofectamine® 2000 Reagent, aprotinin and leupeptin were purchased from Invitrogen. The pMD19-T was obtained from Takara. T4 DNA ligase and two restriction enzymes *Kpn*I and *Xho*I were purchased from MBI Fermentas Company. RIPA lysis buffer and BCA protein assay kit were supplied by Beyotime Institute of Biotechnology (Jiangsu, China). Standard rosuvastatin and finasteride were supplied by Zhejiang Donggang Pharmaceutical Company (Taizhou, China). All solvents used were HPLC grade and all chemicals were analytical grade.

4.2. Methods

4.2.1. Bioanalytical method

Concentrations of rosuvastatin in cell lysatea were determined by a ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS). The assay procedure involved a simple deproteinisation of cell lysate (100 µL) by precipitation with 200 µl of finasteride (internal standard) acetonitrile solution (100 nM), followed by centrifugation (17000× g 10 min) and injection of the supernatant (7 µL) into the UPLC-TQD system (Waters, Milford, MA, USA). Chromatographic separation was achieved on an X-bridge C_{18} column (3.5 µm, 2.1×50 mm) with a mobile phase consisting of a gradient mixture of methanol (A) and ammonium acetate 5 µmol/l in water (B) at a flow rate of 0.25 ml/min. The gradient program of the mixture ratio of A and B was set as follows: 70% B for 0–1 min, linear gradient from 70 to 25% B for 1–1.2 min, 25% B for 1.2–3.0 min. Detection was performed by positive ion electrospray ionization in multiple reaction monitoring mode (m/z 482.4→258.3 for rosuvastatin and m/z 373.5→305.5 for internal standard), with a capillary voltage of 3.8 kV, a source temperature of 150°C, a desolvation temperature of 300°C, a cone voltage of 62 V and collision energy of 30 eV. Rosuvastatin was quantitated over the concentration range of 25 to 150 nmol/l, with good intra- and inter-day precision (RSD≤10%, n=5).

4.2.2. Construction of OATP1B1*1a, OATP1B1*1b and OATP1B1*15

The OATP1B1 cDNA was cloned from human liver total RNA using the RT-PCR method. Primers of specific for OATP1B1 were designated on the basis of the sequence information of NM_006446. The OATP1B1 cDNA was amplified using the forward primer-containing *Kpn*I site, 5'-GGGGTACCATCATGGACCAAAATCAAC-3', and the reverse primer-containing *Xho*I site, 5'-CTCGAGTGGAAACACAGAAGCAGAAG -3'. The PCR product was ligated into the pMD19-T vector followed by sequencing. Full length OATP1B1 was cut from pMD19-T using *Kpn*I and *Xho*I, and subcloned into pcDNA3.1/(+) (Invitrogen).

4.2.3. Cell culture and the stable cell lines selection

The HEK293 cells were cultured in complete medium consisting of DMEM with 10% fetal bovine serum at 37 °C in a 95% air, 5% CO_2 atmosphere. The constructs (pcDNA3.1/(+)-OATP1B1*1a/*1b/*15) were transfected into HEK-293 cells using Lipofectamine™ 2000 transfection reagent. After transfection, cells were selected with 600 µg/ml G418 for two weeks. Then, G418-resistant colonies were screened by transport study and immunoblot analysis.

4.2.4. RT-PCR analysis of OATP-C mRNA expression

Total RNA was extracted from HEK-OATP1B1 and HEK-Control cells using Trizol reagent. A 2 µg-portion was primed with oligo-dT and reverse-transcribed using M-MuLv Reverse Transcriptase. Primers for amplification of OATP1B1 cDNA were as follows: OATP1B1-F: 5'-CAATAGAGCATCACCTGAGATAGTGG-3' (sense) and OATP1B1-R: 5'-CGAGAATCAGTAGGAGTTATCCTGATAG-3'(antisense). Human β-action gene was used as an inner-reference. PCR cycling conditions were as follows: 5 min denaturation at 98 °C; 30 cycles of 10 *s* at 98 °C, 15 *s* at 53 °C, 2 min at 72 °C; 10 min final elongation at 72 °C. RT-PCR products were resolved on a 1.5% agarose gel at 90V for 30 min, visualized by ultraviolet illumination. A 309-bp fragment of OATP1B1 cDNA and a 550-bp fragment of β-action were amplified using specific primers.

4.2.5. Preparation of membrane fractions

HEK-OATP1B1 and HEK-Control cells were disrupted by sonication in RIPA lysate. After centrifugation (100,000g; 4 °C; 45 min), pellets were resuspended in Tris buffer (50 mM; pH 7.4). The membrane fractions were stored at -70 °C before being used for Western blot analysis. All membranes were prepared in the presence of protease inhibitors (aprotinin 10 g/mL, leupeptin 10 µg/mL) and all procedures were performed at 0–4 °C. The membrane protein concentration was determined by BCA assay.

4.2.6. Western blotting

The membrane proteins (50 µg) were separated on 8% resolving sodium dodecylsulfate (SDS)-polyacrylamide gels, followed by electrophoretic transfer to PVDF membrane for 90 min. Then, the membrane was blocked in 5% BSA in TBS-T overnight at 4 °C. This was followed by incubating with 300-fold diluted OATP1B1 antibody in 2.5% BSA in TBS-T for 2 h at room temperature. After five washes with TBS-T, the membrane was incubated with 7500-fold diluted goat anti-rat IgG(H+L) for 60 min followed by another three washes with TBS-T. The signals were detected using ECL™ Western Blotting Detection Reagents.

4.2.7. Uptake transport assays

HEK-OATP1B1 and HEK-Control cells were seeded in 12-well dishes. After 72 h, uptake experiments were initiated after cells were washed once with 37 °C HBSS and preincubated with HBSS containing 25 µM rosuvastatin at 37 °C for 30 min. At the end of the experiment, cells were washed with ice-cold PBS buffer and lysed with 100 µL of 0.2% SDS. The intracellular rosuvastatin accumulation was determined by the UPLC-MS-MS. Protein concentration was determined with BCA assay. Rosuvastatin uptake in each well, expressed as nmol/mg protein/min, was calculated as intracellular accumulation of rosuvastatin within a given time period divided by protein content.

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Author contribution: ZHOU Q and ZENG S conceived and designed research; Chen M, Qu BX and Chen XL performed molecular experiments and analyzed samples; Hu HH provided guidance on molecular technologies; Jiang HD and Yu LS performed data analysis; and Quan ZHOU and Su ZENG wrote the paper.
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