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Short Communication

Hepatocyte Nuclear Factor 1 Alpha is a Factor Responsible for the Interindividual Variation of OATP1B1 mRNA Levels in Adult Japanese Livers

Tomomi Furihata,[1,2] Tomomi Satoh,[1] Naoko Yamamoto,[1] Kaoru Kobayashi,[1] and Kan Chiba[1]

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Purpose. The aim of the present study was to clarify the factors responsible for interindividual variability of organic anion transporting polypeptide (OATP, gene symbol *SLCO*) 1B1 mRNA expression level in the human liver.
Materials and Methods. OATP1B1 mRNA expression levels were determined by real-time PCR in 31 human liver samples. The results were analyzed in relation to a single nucleotide polymorphism (−11187G>A) located in the promoter region and levels of hepatocyte nuclear factor (HNF) 1α mRNA.
Results. There was a 4.9-fold interindividual variability of OATP1B1 mRNA expression level in the livers analyzed, which was not associated with −11187G>A polymorphism. Accordingly, the −11187G>A polymorphism did not alter the *SLCO1B1* gene promoter activity in luciferase assays. On the other hand, OATP1B1 mRNA levels showed a significant correlation with HNF1α mRNA levels (r=0.83, P<0.0001). This correlation was consistent with the results of luciferase assays and chromatin immunoprecipitation assays showing functional interaction between HNF1α and *SLCO1B1* gene promoter.
Conclusions. Our results suggest that HNF1α is an essential regulator of OATP1B1 mRNA expression and thus the level of HNF1α expression is one of the major determinants of interindividual variability in OATP1B1 mRNA expression.

KEY WORDS: hepatocyte nuclear factor 1α; interindividual variability; liver; organic anion transporting polypeptide 1B1; transcriptional regulation.

INTRODUCTION

Human organic anion transporting polypeptide 1B1 (OATP1B1, gene symbol *SLCO1B1*) is specifically expressed in the liver, where it mediates basolateral uptake of various compounds from circulation. Structurally diverse drugs, such as HMG-CoA reductase inhibitors and rifampicin, have been reported to be its substrates, making OATP1B1 one of the major determinants for hepatic disposition of substrate drugs (1). OATP1B1 also contributes to liver physiological functions by transporting endogenous organic anions, such as bile salts, bilirubin conjugates, thyroid hormones, and eicosanoids (1,2). Therefore, gain or loss of transport activity of OATP1B1 in individuals could have a great impact on their pharmacokinetic profiles of substrate drugs and physiological homeostasis.

It is thought that there are two main reasons for the interindividual variability of the level of transporting activity of OATP1B1: (1) existence of genetic amino acid variation at a functionally important position in the polypeptides and (2) increased or reduced level of expression of the *SLCO1B1* gene. With respect to the first reason, several single nucleotide polymorphisms (SNPs) have been characterized. One of them, 521T>C (174V>A), has been shown to impair membrane localization of OATP1B1 in cultured cells, eventually resulting in remarkable loss of the function of the transporter (3). Consistent with this *in vitro* finding, it has been reported that the carriers of this SNP showed significant increase in plasma pravastatin concentrations (4,5). This SNP, together with 388A>G (130N>D), has also been reported to be one of the genetic factors related to unconjugated hyperbilirubinemia (6). On the other hand, with respect to the second reason, some researchers have shown that hepatocellular carcinoma and primary sclerosing cholangitis tend to decrease the level of *SLCO1B1* gene expression (7–10). More recently, Ho *et al.* (11) have examined the expression profiles of *SLCO1B1* and *SLCO1B3* genes in 22 human livers, the results of which suggest the existence of interindividual variability in the level of *SLCO1B1* gene expression. The mechanisms by which *SLCO1B1* gene transcription is regulated likely play some roles in the variability of

Tomomi Satoh and Naoko Yamamoto equally contributed to this work.

[1] Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, 260-8675, Japan.
[2] To whom correspondence should be addressed. (e-mail: tomomif@p.chiba-u.ac.jp)

ABBREVIATIONS: HNF, Hepatocyte nuclear factor; MODY, Maturity-onset diabetes of the young; OATP, Organic anion transporting polypeptide; PCR, Polymerase chain reaction; SLCO, Solute carrier organic anion transporter; SNP, Single nucleotide polymorphism.

expression levels. Hepatocyte nuclear factor (HNF) 1α has been demonstrated to be a strong activator of the *SLCO1B1* gene promoter in HepG2 cells (12). The SNP (−11187G>A) has been identified within the promoter region of the *SLCO1B1* gene, and the associations of the SNP with increased pravastatin concentration and blood glucose response to repaglinide have also been reported (4,13). However, *in vivo* contribution of HNF1α to *SLCO1B1* gene transcription has not been clarified and other factors that are involved in *SLCO1B1* gene transcription remain unidentified. Effects of the SNP (−11187G>A) on expression level of the *SLCO1B1* gene has been unclear. Therefore, the factors causing the variability in *SLCO1B1* gene expression remain unknown.

In the present study, we determined the levels of OATP1B1 mRNA expression in liver samples obtained from 31 Japanese patients who had undergone hepatectomy. To identify the causal factors for the interindividual variability, we analyzed the relationships of OATP1B1 mRNA expression level with SNP (−11187G>A) and expression level of HNF1α mRNA.

MATERIALS AND METHODS

Human Liver Tissues

Liver tissues were obtained from the National Cancer Center Hospital East (Kashiwa, Japan) and from the International Medical Center of Japan (Tokyo, Japan). The liver samples were normal parts surrounding tumor areas that had been surgically resected from patients with hepatocarcinoma. The hepatocarcinomas in patients resulted from metastasis from colorectal or gastric cancer. The liver tissues were snap-frozen in liquid nitrogen and stored at −80°C or in liquid nitrogen until used. Liver information is shown in Table I. Non-frozen liver tissues (Liver I and Liver II) were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Tokyo, Japan). Liver I was from an American-African female patient (34 years old), and Liver II was from a Hispanic female patient (35 years old). These liver tissues had been rejected for liver transplantation. All of the patients were negative for hepatitis B virus and hepatitis C virus. Full permission was obtained from the Ethics Committee of Chiba University, Japan, based on the Helsinki declaration.

Table I. Characteristics of Japanese liver samples

Number	Gender	Age (years)
401	Female	76
501	Female	52
601	Male	64
901	Male	49
1001	Male	67
1101	Male	67
1301	Male	71
1401	Male	64
1801	Male	54
2201	Male	56
2301	Male	70
2401[a]	Female	29
2501	Female	71
2601	Male	75
2701	N/A	N/A
2801	N/A	N/A
2901	Male	62
3001	Male	47
0	N/A	N/A
1	Male	60
3	Male	N/A
19[a]	Male	51
20[a]	Male	56
21	Male	59
26	Male	62
34[a]	Female	44
36	Male	53
38	Female	55
39	Male	64
40[a]	Male	64
42	Male	62

Hepatic metastasis from colorectal or gastric cancer was existed in all patients.
N/A, data not available.
[a] Heterozygote carriers of −11187G>A.

Total RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (real-time PCR)

Total RNAs were extracted from liver tissues by using an SV Total RNA Isolation System (Promega, Madison, WI). Quality of total RNA was assessed by determination of the ratio of 28S rRNA to 18S rRNA by densitometric analysis. No genomic DNA contamination was confirmed by performing PCR as described previously (14). The method of cDNA synthesis was described elsewhere (14).

Real-time PCR was performed by using Taqman Gene Expression Assays with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City CA) as described previously (14). The probe and primers were Hs00272374-m1 (*SLCO1B1*) and Hs00167041-m1 (*HNF1α*). Four house-keeping genes, beta-glucronidase, cyclophilin, acidic ribosomal protein and glyceraldehyde-phosphate dehydrogenase, were selected as internal standards based on the results of preliminary tests using a Taqman Human Endogenous Control Plate (Applied Biosystems) and previously reported methods in which the levels of expression of target genes were normalized by the averaged value of multiple internal standard genes (15–17). The data were expressed as arbitrary units with the lowest value set to 1. All assays were performed at least three times in triplicate, and analyses of most samples were performed twice with independent total RNA isolation, reverse transcription and real-time PCR.

Genomic DNA Isolation and DNA Sequencing for Genotyping

Genomic DNA was isolated from liver tissue by using a Charge Switch gDNA Micro Tissue Kit (Invitrogen, Carlsbad, CA). An SNP of the *SLCO1B1* gene (−11187G>A, translation start codon=+1) was analyzed by PCR and DNA

sequencing. The primers used for PCR were 5′-CCCATGAA TGATAAGGGGTAACCATA (sense) and 5′-ACTTGACT TGTGGAGAAAGGAAGAGC (anti-sense). The procedure used for DNA sequencing was described previously (18).

Luciferase Reporter Plasmid Construction and Site-directed Mutagenesis

The 2068-bp 5′-upstream region of the *SLCO1B1* gene was cloned from genomic DNA prepared as described above by PCR with the primers 5′-CCTGAGAACTCTGGGGC TAAAACCT (sense) and 5′-AAGACCATCAAAATCT TCTTCCGA (anti-sense). The obtained genomic DNA fragment was inserted into a pCR-Blunt II TOPO vector (Invitrogen), and the DNA sequence was analyzed. Then the upstream region was transferred to a pGL3-basic vector (Promega). This wild-type reporter construct was named pSLCO1B1/wt. To obtain a reporter plasmid harboring −11187G>A, site-directed mutagenesis was performed by methods described previously (19) with the primers 5′-TGTGCATATGTGTATACAAGTAAAAGTGTGTAT (sense) and 5′-ATACACACTTTTACTTGTATACACATA TGCACA (anti-sense). The underlines indicate the position of −11187G>A. The reporter construct harboring a mutation was named pSLCO1B1/mt. The mammalian expression vector of human HNF1α was kindly provided Dr. Ryuichirou Satoh (Lab. of Food Biochemistry, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo).

Cell Culture, Transient Transfection and Dual Luciferase Assay

HepG2 cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin.

Transient transfection was performed as described previously (14) with 200 ng/well of the reporter plasmid, 4 ng/well of phRL-TK (Promega). Either HNF1α expression plasmid (pHNF1α) or pTarget empty plasmid (50 ng/well) was added to the transfection mixture in a cotransfection assay. After 24 h of incubation, the cells were harvested and the luminescence was determined as described previously (20).

Preparation of Nuclear Extracts from Hepatocytes and Gel Mobility Shift Assay (GMSA)

Nuclear extracts were prepared from HepG2 cells and primary hepatocytes as described previously (19). The primary hepatocytes were prepared from the non-frozen liver tissue described above at the Human and Animal Bridging Research Organization, and they were immediately used for nuclear extraction. Protein concentration was determined by using a Bio-Rad Dc Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

The methods used for GMSA were described in detail in our previous report (14). Oligonucleotide sequences containing wild-type or mutant nucleotides are 5′-TGTGCATAT GTGTATACAGGTAAAAGTGTGTAT (wild type) and 5′-TGTGCATATGTGTATACAAGTAAAAGTGTGTAT (mutant). Only sense strands are shown.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed by using a ChIP-IT kit (Active Motif, Carlsbad, CA). The method for preparation of sheared chromatin from the human liver (Liver I and Liver II) was based on the report of del Castillo-Olivares *et al.* (21). A piece of human liver (800 mg) was isolated and chopped on ice. Cross-linking of proteins to DNA was done by adding 1% formaldehyde in PBS to the chopped liver for 12 min with gentle rotation. To stop the reaction, an equal volume of 250 mM glycine in PBS was added to the mixture for 5 min with rocking. The tissue debris was collected by centrifugation at 2,500 g for 3 min and washed with PBS twice. The pellet was resuspended with PBS containing phenylmethylsulfonyl fluoride and homogenized by a dounce homogenizer. After centrifugation at 2,500 g for 10 min, the sample was resuspended in the lysis buffer supplied with the ChIP-IT kit. The chromatin was sheared by using an Ultrasonic disrupter UD-201 (TOMY, Tokyo, Japan) at 25% power with six pulses. For immunoprecipitation, 12 μg of the sheared chromatin was mixed with either 3 μg of control goat antibodies (sc-2028, Santa Cruz Biotechnology, CA, USA) or 3 μg of anti-HNF1α IgG (sc-6547x, Santa Cruz Biotechnology). After incubating for 12 h at 4°C with gentle rotation, the following procedure was used as described in a previous report (14). PCR was performed with two sets of primers at 38 cycles. One set of primers was for detection of the region containing the HNF1α binding site in the *SLCO1B1* promoter, 5′-TGGCAACTGGAGTGAACTCTTAAAAC (sense) and 5′-TCCTACAGCAACTGCAACAAGTCCAC (antisense), and the other set of primers was for detection of the intronic region between exons 8 and 9, 5′-CCTTCTACTCCTTATTT CAAGCAG (sense) and 5′-ACTGAGAGTTGGATGA TACGGACT (antisense).

Statistical Analysis

The difference between OATP1B1 mRNA expression levels in carriers of the SNP and non-carriers was analyzed by the Mann–Whitney U test. The correlation of levels of OATP1B1 mRNA with those of HNF1α mRNA was analyzed by linear regression analysis. These statistical tests were performed by using Statcel software (OMS Publishing Inc., Tokyo, Japan).

RESULTS AND DISCUSSION

Real-time PCR was performed to determine the level of variability in OATP1B1 mRNA expression in the human liver. The results showed that there was an approximately 4.9-fold difference in the expression level. To identify the factors causing the interindividual difference in the OATP1B1 mRNA expression level, we analyzed the effects of the SNP (−11187G>A) on OATP1B1 mRNA expression level. The SNP was located in the promoter region of the *SLCO1B1* gene, 814 bp upstream from the transcription start site (11187 is the position with respect to the translation start

codon). The SNP has been reported to be associated with increased pravastatin concentration and blood glucose response to repaglinide (4,13), possibly because the SNP may affect the level of *SLCO1B1* gene expression. However, whether the associations are attributed to the effect of the SNP on expression of OATP1B1 mRNA has been controversial due to partial linkage between −11187G>A and 521T>C, the latter of which has been shown to impair the function of OATP1B1 protein. Thus, we analyzed the effects of the SNP on the expression levels of OATP1B1 mRNA by comparing the mRNA levels in G/G (wild-type) homozygotes and G/A heterozygotes of −11187G>A. As shown in Fig. 1A, there was no significant difference between the two groups. We also examined the effect of the SNP on promoter activity by luciferase reporter assays. Consistent with the result described above, the SNP failed to alter promoter activity of the *SLCO1B1* gene in HepG2 cells (Fig. 1B). In addition, we performed GMSA, the results of which showed that the SNP did not affect the binding profile of nuclear proteins prepared from HepG2 cells or human hepatocytes to the oligonucleotides (data not shown). These results suggest that the SNP (−11187G>A) is not functional and that the apparent associations of the SNP with drug pharmacokinetics or response are mainly a result of the effect of 521T>C. Taken together, our results suggest that the SNP (−11187G>A) is unlikely to be a major determinant of OATP1B1 mRNA expression level at least in Japanese livers.

Although little is known about the mechanisms controlling *SLCO1B1* gene transcription, it has been reported that HNF1α is a strong activator of the *SLCO1B1* promoter (12). We confirmed that HNF1α could strongly enhance *SLCO1B1* promoter activity (18-fold increase) in luciferase assays (data not shown). Then, we further performed chromatin immunoprecipitation assays by using two human liver tissues (Liver I and Liver II) to examine *in vivo* binding of HNF1α to the *SLCO1B1* promoter. The results showed that amounts of the *SLCO1B1* promoter region in DNA fragments precipitated with anti-HNF1α antibodies were larger than those in fragments precipitated with control antibodies (Fig. 2), indicating that HNF1α can bind to the promoter region of the *SLCO1B1* gene *in vivo*. Collectively,

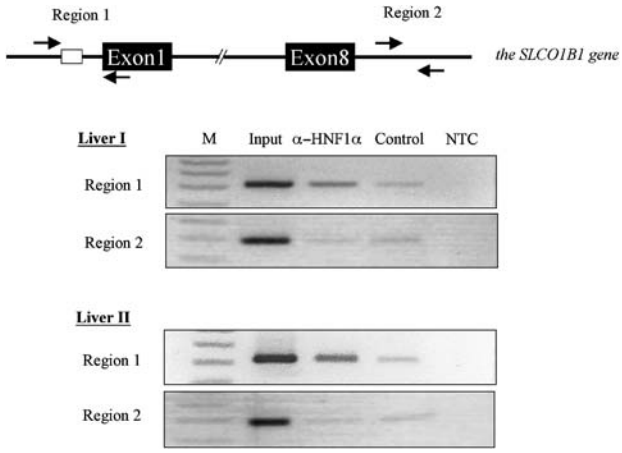

Fig. 2. Interaction between HNF1**α** and *SLCO1B1* gene promoter. Binding of HNF1α to *SLCO1B1* gene promoter region in the human liver examined by chromatin immunoprecipitation assay. Two human liver tissues (*Liver I* and *Liver II*) were used in this assay. Sheared chromatin was incubated with either control goat antibodies (Control, 3 μg) or anti-HNF1α antibodies (α-HNF1α, 3 μg) for 12 h at 4°C. *Arrows* indicate the positions of primers, and a *white box* indicates an HNF1α binding site. PCR cycles were 38. *M*, DNA marker; *NTC*, non-template control.

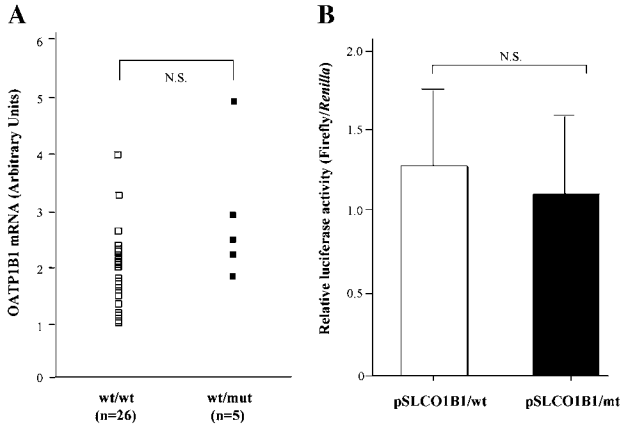

Fig. 1. Effects of the SNP (−11187G>A) on expression level of OATP1B1 mRNA. **A** comparison of OATP1B1 mRNA levels in carriers and non-carriers (*wt/wt*, −11187GG homozygote; *wt/mut*, heterozygote of −11187G and A). Expression levels of OATP1B1 mRNA were determined by real-time PCR. *N.S.*, not significant (Mann–Whitney *U* test). **B** effects of an SNP (−11187G>A) on promoter activity of the *SLCO1B1* gene in HepG2 cells. Results are expressed as means ± S.D. of three experiments, each performed in triplicate. *N.S.*, not significant (Student's *t* test); *pSLCO1B1/wt*, pGL3-Basic vector carrying wild-type 2068 bp of the 5′-upstream region of the *SLCO1B1* gene; *pSLCO1B1/mt*, pGL3-Basic vector carrying 2068 bp with the mutation of −11187G>A.

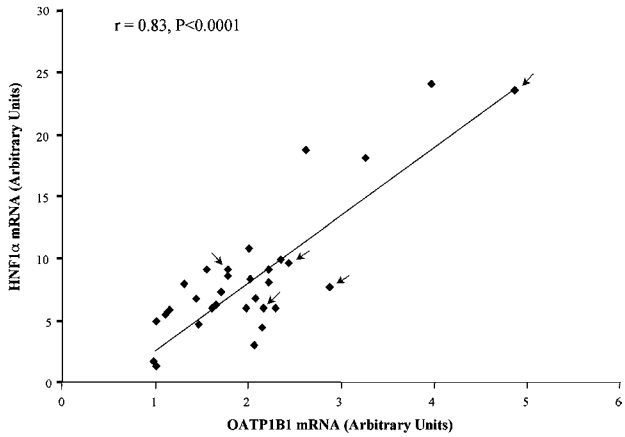

Fig. 3. Correlation between OATP1B1 mRNA expression levels and HNF1**α** mRNA expression levels in human liver samples. The expression levels of OATP1B1 mRNA and HNF1**α** mRNA levels in liver samples (n=31) were determined by real-time PCR. Results were normalized by using the mean value of four human endogenous genes (see Materials and Methods). Arbitrary units were calculated with the lowest level of mRNA (# 2801 for OATP1B1 and #1001 for HNF1α) set to unit=1. The values are expressed as means ± S.D. of three separate determinations. Correlation was analyzed by linear regression analysis. *Arrows* indicate the samples carrying the SNP −11187G>A.

HNF1α would play an important role in the regulation of *SLCO1B1* gene transcription in human liver.

Since HNF1α has been shown to be an important factor regulating *SLCO1B1* gene transcription, we examined the association of HNF1α with OATP1B1 mRNA expression in the human liver. As shown in Fig. 3, the levels of HNF1α mRNA varied by 23.8 fold in the samples, and the levels of HNF1α mRNA showed a significant correlation with OATP1B1 mRNA levels (r=0.83, p<0.0001). This correlation was unlikely due to the quality of RNA tested because when compared with UDP-glucuronosyltransferase 1A1 and 1A3 mRNA levels, OATP1B1 mRNA levels were not correlated with either gene (unpublished observation). This finding together with the findings described above suggest that HNF1α is an essential regulator of OATP1B1 mRNA expression and thus the level of HNF1α expression is one of the major determinants of the interindividual variability in OATP1B1 mRNA expression.

It is known that several mutations of the HNF1α gene are responsible for maturity-onset diabetes of the young type 3 (MODY3). MODY3 has been shown to be the most common type of MODY in the United Kingdom and a common type of MODY in German, Danish, Italian, Finnish, North American, and Japanese pedigrees (22,23). It is thought that impaired function of HNF1α caused by a mutation leads to aberrant expression of its target genes. Thus, the expression level of OATP1B1 mRNA may be decreased in patients with MODY3, and pharmacokinetics of drugs transported by OATP1B1 might be altered in such patients. Although there has been no study in which the levels of OATP1B1 expression in the livers of MODY3 patients were examined, it has been reported that *Hnf1α*$^{-/-}$ mice, a model for MODY3, showed lower hepatic uptake of glibenclamide than that in wide-type mice, resulting in higher plasma concentration of the drug (24). Although the murine hepatic glibencramide transporter has not been identified, it has been shown that some hepatic uptake transporters are downregulated in *Hnf1α*$^{-/-}$ mice (25,26). Therefore, it may be worth evaluating the possibility of alteration in the *SLCO1B1* gene expression level or drug disposition in MODY3 patients.

In conclusion, our results show that the expression level of HNF1α but not the SNP (−11187G>A), is a strong candidate for factors causing the variability of OATP1B1 mRNA level observed in human livers. Since it has been reported that HNF1α can interact with various transcription factors, it is likely that HNF1α orchestrates multiple factors on the *SLCO1B1* gene to control its transcription. Such factors are currently unknown, and further study is needed to determine the factors and how they cooperate with HNF1α. The results of such study would reveal the impact of HNF1α on *SLCO1B1* gene transcription, which is one of the cornerstones for further understanding the interindividual variability of OATP1B1 mRNA expression in a steady state as well as in pathological conditions.

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