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Characterization of six base pair deletion in the putative HNF1-binding site of human *PXR* promoter

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Abstract Pregnane X receptor (PXR) regulates transcription of drug metabolism genes such as *CYP3A4* and *MDR1*. Several species of *PXR* transcripts have been reported, including *hPAR-2* with an extended amino-terminus. Database search identified a 6-bp deletion at the putative HNF1 binding element on the proximal region flanking to the *hPAR-2* transcription start site. Aspirin-induced asthma (AIA) is a typical drug-induced phenotype due to aspirin or nonsteroidal antiinflammatory drugs, and these drugs are metabolized by CYP2C9 and UGT1A6, which are regulated by PXR. We examined a possible association between the 6-bp

deletion variant and AIA; 129 AIA patients and 117 controls were genotyped, and no allelic association was observed. Characterization of the *hPAR-2* promoter revealed that the proximal region of 1.5-kb from the transcription start site conferred a promoter activity and that the 6-bp deletion diminished the activity. These results suggest that the putative HNF1 binding element is essential for the transcriptional activity of *hPAR-2* and also, that substantial numbers of Japanese are in a deficient state. Because of the biological significance of the 6-bp deletion of *PXR*, the variant might potentially associate with as yet unknown phenotype.

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Introduction

Pregnane X receptor (PXR) is one of the orphan nuclear receptors and comprises the NR1I subfamily of nuclear receptors with other members, constitutive androstane receptor, farnesoid X-activated receptor, and vitamin D receptor. PXR is predominantly expressed in the liver and intestines, and has DNA- and ligand-binding domains. There are an increasing number of xenobiotics that bind the ligand-binding domain, such as rifampicin, dexamethasone, and the herb St. John's wort (Lehmann et al., 1998; Blumberg et al., 1998; Bertilsson et al., 1998; Jones et al., 2000; Moore et al., 2000; Wentworth et al., 2000). Upon ligand binding, the activated PXR forms a heterodimer with retinoid X receptor, and the dimer binds to consensus sequences (i.e., DR-3, DR-4, ER-6, or ER-8) in regulatory regions of the downstream genes (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998). HNF4 α involves in this PXR-mediated transcriptional activation (Kamiya et al., 2003; Tirona et al., 2003). PXR regulates transcription of genes important for drug metabolism and disposition such as *CYP3A4* (Lehmann et al., 1998; Goodwin et al., 1999) and *MDR1* (Geick

et al., 2001), and for bile acid metabolism such as *CYP7A1* (Staudinger et al., 2001a; Staudinger et al., 2001b), *MRP2* (Kast et al., 2002), and *Oatp2* (Staudinger et al., 2001a). Bile acids are essential for absorbing lipids and fat-soluble vitamins, and provide an important pathway for eliminating excess cholesterol from the body.

In addition to the transcript originally reported (Lehmann et al., 1998; Blumberg et al., 1998; Bertilsson et al., 1998), which we call *hPXR* here, other transcript species have been identified in humans, including *hPXR.2* (Dotzlaw et al., 1999), *hPAR-2* (Bertilsson et al., 1998), and several others (Hustert et al., 2001; Zhang et al., 2001; Fukuen et al., 2002). *hPXR* and *hPXR.2* cDNAs do not contain classical ATG initiation codon but instead initiates at an alternative CTG codon, as indicated in Fig. 1A. *hPAR-2* has a longer N-terminus than the *hPXR* and *hPXR.2* due to an alternative use of exon 1b instead of exon 1a, suggesting a possibility that *hPAR-2* is transcribed from an alternative promoter. Although the *PXR* promoter has been characterized in

the mouse (Kamiya et al., 2003), the promoter of the gene remains to be examined in human cells.

Aspirin-induced asthma (AIA) affects approximately 10% of adult asthmatics and is well known as an acute symptom to be precipitated by oral administration of aspirin or other nonsteroidal antiinflammatory drugs (NSAIDs). Because AIA is a typical drug-induced phenotype, and aspirin and NSAIDs are metabolized by CYP2C9 and UGT1A6, which are regulated by PXR, the molecular variant of *PXR* could possibly participate in the molecular pathogenesis of AIA. We detected a 6-bp deletion in the promoter region of *hPAR-2* through the database search, which possibly affects the rate of transcription. Then allelic association of the 6-bp deletion with AIA was tested, and the functional impact of the deletion was examined by in vitro reporter assay.

Materials and methods

Plasmid preparation

To identify a proximal transcriptional regulatory region of *hPAR-2*, a transcript species of *PXR*, its cDNA sequence deposited in GenBank (accession #NM_022002), was used for a homology search using the Blast program at the National Center for Biotechnology Information (NCBI). Because 5'-end sequence was determined by 5' RACE (Bertilsson et al., 1998), the 5'-end of this cDNA sequence indicates a transcription start site. Human genomic sequence (GenBank # NT_005612) was used to design PCR primers to amplify the 0.4-, 0.8-, 1.5-, 2.8-, and 3.9-kb segments from the transcription start site of *hPAR-2*. Primers were designed with the Primer Express version 1.0 (Applied Biosystems). To facilitate the subsequent subcloning of the PCR products, each forward and reverse primer contained flanking sequence of *Kpn* I and *Hind* III recognition site, respectively. Primers in this study are listed in Table 1. PCR was carried out in a 20- μ l PCR reaction containing 1 X *ExTaq* buffer (TaKaRa Biomedical), 2.5 mM dNTPs, 2 U of *ExTaq* polymerase (TaKaRa Biomedical), and 10 ng of human genomic DNA as a template, and the thermal cyclor condition was 95°C, 30 s; 55°C, 45 s; and 72°C, 1.5 min. The reaction was carried out for 30 cycles. The PCR products were

Fig. 1A–C Characterization of *PXR* (*hPAR-2*) promoter. **A** Structure of human *PXR* transcripts. Exons are depicted by an open box and each exon is numbered. Initiation and termination codons are indicated by ATG/CTG and TGA, respectively. *hPAR-2* uses exon 1b, an alternative to exon 1a that *PXR* and *hPXR.2* uses. *hPXR.2* contains an in-frame deletion due to a preferential usage of a cryptic splicing acceptor site of exon 5. The location of 6-bp deletion variant was shown with ($\downarrow$). **B** Promoter activities on the upstream sequence of *hPAR-2* were determined. The segments of 0.4–3.9-kb relative to the transcription start site were amplified by PCR and subcloned into the promoterless pGL3 basic vector. The constructs were transfected into HepG2 cells and the firefly luciferase activity was normalized with the *Renilla* luciferase activity of cotransfected pRL-TK vector. An arrow indicates the location of the firefly luciferase gene. **C** The effect of the 6-bp deletion of the putative HNF1 binding site on the *hPAR-2* promoter activity. The 1.5-kb constructs with or without the deletion were cloned and used for luciferase assay as described before. An arrowhead indicates the location of the 6-bp deletion

Table 1 Primer sequence used for preparation of luciferase reporter constructs. Forward (F) and reverse (R) primers contained restriction enzyme recognition sites for *Kpn* I and *Hind* III, respectively, at the 5' end. The reverse primer was used for preparation of all constructs

Primer sequence 5' → 3'	Construct
F-CACTGAACTGAGCAGCCACCAAATGCAAT	hPAR2/-3.9k
F-GGCAAAGTATAGAATGGAGGTCAAGGCCTCA	hPAR2/-2.8k
F-ACTTCCACTCCCCTCACCTGCCATAAAGCA	hPAR2/-1.5k
F-CTGGACTTGGGACTTAGGAGGGGCAATGGA	hPAR2/-0.8k
F-CATAGTGGGTCGTGAATCATGTTGGCCTTG	hPAR2/-0.4k
R-GGCCTCAGGGGGCTGCCAGCACTAACCCAG	

subcloned into the pGL3 basic vector (Promega) using *Hind* III and *Kpn* I sites. The inserts of the clones were verified by sequencing.

Transient transfection assays

A human hepatoblastoma cell line, HepG2 (Riken), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen). The cells were plated at 5×10^4 cells/well onto a 24-well plate and grown for 24 h. Then transfection was performed with 20 ng of each pGL-3 construct and pRL-TK vector (Promega) using FuGENE 6 (Roche) according to the manufacturer's instructions. After 48 h, cells were lysed and assayed using a dual luciferase assay kit (Promega) according to the manufacturer's protocol. The firefly and *Renilla* luciferase activities were measured by an AutoLumat LB953 (Berthold Systems) and firefly luciferase activities were normalized for transfection efficiency using the activity of pRL-TK vector. Each experiment was repeated at least three times to minimize the experimental bias, and all measurements were averaged to determine a representative value for each experiment.

Patients and genotyping

AIA patients were recruited in Niigata University and Nagoya University; 129 patients (mean age 57.3 ± 12.5) were diagnosed by clinically documented asthmatic responses after ingestion of aspirin or NSAIDs. As controls, 117 unrelated individuals (mean age 75.0 ± 6.9 years) free of asthmatic episode were recruited in Niigata and Aichi Prefectures. The study was approved by the IRB of Niigata University and Nagoya University. Genomic DNAs of patients and controls were subjected to PCR amplification followed by direct sequencing using BigDye terminator cycle sequencing and ABI 3700 DNA analyzer (Applied Biosystems).

Results and discussion

Database search using the JSNP (http://snp.ims.u-to-kyo.ac.jp/index_ja.html) identified six SNPs (data not shown) and a 6-bp deletion in *hPAR-2*, a splicing species of *hPXR*. Because the 6-bp deletion is at a putative HNF1 binding site in the promoter region (see Fig. 1a), the deletion appears to have impact on the promoter activity and subsequently affect expressions of downstream genes such as CYP families.

First, we tested whether the deletion variant was associated with AIA, a drug-induced asthma, because aspirin and NSAIDs metabolizing enzymes are under regulation of PXR. We genotyped 129 AIA patients and 117 controls for 6-bp deletion variant. Hardy-Weinberg disequilibrium was maintained for both controls and AIA (data not shown). As shown in Table 2, the allele

Table 2 Allelic association between 6-bp deletion of *PXR* and AIA. + denotes major type; – denotes 6-bp deletion

	AIA <i>n</i> = 129 (frequency)	Control <i>n</i> = 117 (frequency)	
Genotype			
++	64 (0.496)	63 (0.538)	$\chi^2 = 1.321$ df = 2 <i>P</i> = 0.517
+-	57 (0.442)	44 (0.376)	
--	8 (0.062)	10 (0.086)	
Allele			
+	185 (0.717)	170 (0.726)	$\chi^2 = 0.054$ df = 1 <i>P</i> = 0.816
-	73 (0.283)	64 (0.274)	

frequency of the deletion variant was 27.4% for control and 28.3% for AIA, and statistical significance evaluated by chi-square test could not be reached.

Although the deletion variant of *PXR* was not associated with AIA, a possibility still remains that the deletion variant is functional and associates with as yet unknown phenotypes. In order to clarify functional impact of the deletion variant that locates upstream of *hPAR-2* (see Fig. 1A), we determined the active promoter region of *hPAR-2* with a series of promoter segments of 0.4–3.9-kb immediately upstream of the transcription start site of *hPAR-2*. The segments were inserted into a promoterless luciferase reporter vector and used for the subsequent reporter assays in HepG2 cells. Considerable promoter activities were observed with the segments of 0.8–3.9-kb and the further deletion (0.8-kb >) diminished the promoter activity (Fig. 1B). Sequence analysis of the 1.5-kb region using the TRANSFAC database (Genome Net) revealed consensus binding elements for several transcription factors, including liver-enriched HNF1, HNF3 β , and HNF4, all known to be important for various liver functions. This is consistent with the observation that *PXR* is predominantly expressed in the liver.

To examine the effect of the 6-bp deletion on the *hPAR-2* promoter activity, a 1.5-kb promoter segment harbors the 6-bp deletion was compared with the 1.5 kb segment without the deletion. The 6-bp deletion variant completely abolished the promoter activity in HepG2 cells (Fig. 1C), suggesting that the putative HNF1 binding site at this deletion is essential for the activity of *hPAR-2* promoter in HepG2. Taken together, the 6-bp deletion variant most likely renders *hPAR-2*, the splicing species of *PXR*, inactive of promoter activity in liver cells. Because PXR functions as a xenosensor to eliminate external and internal toxic compounds, a loss of the

transcriptional activity of the variant form resulting in less copy numbers of *PXR* transcript possibly leads to reduction in the capability as a xenosensor.

Thus far, gene-mapping of complex disease has not been fully successful despite the benefits of the completion of the Human Genome Project and the SNP database. One reason would be that SNPs and microsatellites usually serve only as markers of the real causalities, and functional impacts of the markers are not investigated. Given these circumstances, some investigators propose for a different kind of genomic technology—so called functional genomics. Accordingly, mining a battery of functional SNPs that harbor biological significance due to the genetic variation would accelerate efficiency of gene mapping of complex traits—especially response to exposures to exogenous molecules like drugs or environmental toxins. Because functional SNPs provide biological information per se, investigators can predict phenotypes in which the SNPs might be involved. We have identified the 6-bp deletion of *PXR*, which was not associated with AIA but was a functional variant reducing the promoter activity of the gene in liver cells. It is notable that about a half of the Japanese population are heterozygous or homozygous for the variant and probably in a deficient state of enzyme activity.

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