Identification and Characterization of Novel Alternative Splice Variants of Human Constitutive Androstane Receptor in Liver Samples of Koreans and Caucasians

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ABSTRACT

Human constitutive androstane receptor (hCAR, NR1I3) is a member of the orphan nuclear receptor family and regulates the transcription of many drug-metabolizing enzymes and drug transporters. Previous studies have shown that the hCAR gene produces a number of different kinds of mRNA splicing variants (SVs) in non-Asian ethnicities. In the present study, we identified 18 hCAR SVs (SV1–SV18), including four novel SVs in Korean human livers. Among the four novel SVs, SV2 showed enhanced transactivation activity when cotransfected with CYP2B6 reporter gene, whereas other SVs were nonfunctional. When profiles of major hCAR SVs were compared among 30 livers from Korean patients and 20 livers from Caucasian patients, the relative composition of each SV showed interethnic variation as well as interindividual variation. The most predominant form of hCAR SV was not wild type, but either SV4 or SV7. The summed relative amounts of SV4 and SV7 ranged from 43.5 to 57.6% in the 30 Korean livers and from 47.2 to 82.6% in the 20 Caucasian livers, suggesting large interindividual variation. The mean relative amount of nonfunctional SV9 was significantly higher in Koreans (29.8%) than in Caucasians (12.8%). The mean relative amount of novel SV2 was 9.7% in Korean livers and 3.5% in Caucasian livers. Expression profiling of hCAR proteins in human livers also supported large interindividual variation in the expression ratio of wild-type and SVs. Our results describe for the first time the direct comparison of hCAR SV profiles between Koreans and Caucasians. The functional relevance of these interindividual and interethnic variations of hCAR mRNA expression needs to be further characterized.

Introduction

The constitutive androstane receptor (CAR; NR1I3) contributes to the inducible expression of genes involved in the disposition of xenobiotics/endobiotics, including CYPs 2B, 2C, and 3A (Wei et al., 2000; Maglitch et al., 2002), UDP glucuronosyltransferases (Sugatani et al., 2005), transporters (Kast et al., 2002), and others (Kakizaki et al., 2003). It has been shown that the inducers of CYP2B6 and CYP3A4 genes regulate, via human CAR (hCAR) in human primary hepatocytes and cell lines, the induction of CYP2B6 expression by phenobarbital and other drugs (Trameli et al., 2000; Maglitch et al., 2003; Wang et al., 2003). Large interindividual variability exists in the hepatic levels of CYP2B6 mRNA and protein; moreover, substantial interindividual differences with respect to expression of hCAR have been reported (Chang et al., 2003; Lamba et al., 2003). To understand the underlying mechanism for the interindividual variability of hCAR expression or hCAR activity, genetic variations in the hCAR gene have been screened in some populations. Through genetic studies, it has been generally agreed that nonsynonymous polymorphisms of the hCAR gene are rare (Lamba et al., 2005). Meanwhile, several studies have reported the presence of alternative splicing variants (SVs) of CAR transcripts in humans, mice, and rats (Choi et al., 1997). In particular, four hCAR SVs have been repeatedly reported in human livers (Auerbach et al., 2003; Savkur et al., 2003): an isoform with a 12-base pair (bp; four amino acids) insertion in intron 6, an isoform with a 15-bp (five amino acids) insertion in intron 7, an isoform with both of the insertions, and an isoform with deletion of exon 7 (39 amino acids). All these splicing events alter the ligand binding domain of hCAR. Compared with wild-type (WT) CAR.1 protein, these SVs of hCAR have a lower affinity for hCAR binding elements of target genes, decreased transactivation potential, and decreased ability to recruit coactivators (Auerbach et al., 2003; Savkur et al., 2003). A study identified five SVs of rCAR in rat liver tissues (Kanno et al., 2005).

Alternative splicing generates segments of mRNA variability that can insert or delete amino acids, shift the reading frame, or introduce premature termination codons. Up to 59% of human genes generate multiple mRNAs by alternative splicing, and 80% of alternative splicing results in changes in the encoded protein (Modrek and Lee, 2003).

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ABBREVIATIONS: bp, base pair; CAR, constitutive androstane receptor; CITCO, 6-[(4-chlorophenyl)imidazo][2,1-b][1,3]thiazole-5-carboxyl-O-(3,4-dichlorobenzyl)oxime; DEHP, di(2-ethylhexyl)phthalate; hCAR, human constitutive androstane receptor; HepG2, human hepatoblastoma cells; LBD, ligand binding domain; PB, phenobarbital; PBREM, phenobarbital-responsive enhancer module; PCR, polymerase chain reaction; PHY, phenytoin; SV, splicing variant; WT, wild type.
2002), which reveals that it is one source of human proteomic diversity.

Considering the linear correlation between CYP2B6 mRNA levels and hCAR mRNA levels, the SVs of hCAR may be one of the contributors to the interindividual variability observed in CYP2B6 expression. We therefore identified the SVs of hCAR in human livers and characterized their regulatory function of CYP2B6 expression. Here, we report the novel functional hCAR SV with enhanced transactivation activity toward CYP2B6 promoters. Furthermore, we directly compare the profile of hCAR SVs between liver cDNA from Korean and Caucasian subjects to find evidence of interethnic variation in splicing events as well as interindividual variation.

Materials and Methods

Chemicals. Phenobarbital (PB) and 6-(4-chlorophenyl)limidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO), phenytoin (PHY) and di(2-ethylhexyl)phthalate (DEHP) were purchased from Sigma-Aldrich (St. Louis, MO) and BIOMOL Research Laboratories (Plymouth Meeting, PA), respectively. DNA retardation gel for electrophoresis, Lipofectamine 2000 transfection kits, cell culture media, and charcoal-stripped fetal calf serum were purchased from Invitrogen (Carlsbad, CA). Fluorogenic TaqMan primers and probes were synthesized by Applied Biosystems (Foster City, CA). The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). All reagents and chemicals were of the highest grade available from commercial sources.

Human Livers. Human liver tissues were obtained, with informed consent and with institutional review board approval, from the Inje Paik University Review Board, from patients undergoing partial hepatectomy for removal of metastatic tumors at the Department of General Surgery, Busan Paik Hospital (Busan, Korea), as previously described (Liu et al., 2005). Resected tissue samples were histochemically examined, and only histologically normal liver tissues were used. A total of 30 liver samples used for this study are listed in Table 1. Caucasian liver tissues (n = 20) were purchased from XenoTech (Kansas City, KS).

Preparation of Liver cDNA. Total RNA preparation from liver tissue was prepared using the RNase MidiKit (Qiagen, Hilden, Germany) with DNase I treatment according to supplier instructions. RNA integrity was measured using the Agilent Bioanalyzer RNA600 Nano Laboratory Chip Kit (Agilent Technologies, Waldbronn, Germany). Only RNA samples with an RNA integrity number higher than seven were used for further analyses. Each sample was reverse-transcribed using 3 μg of RNA, random hexamers, and the TaqMan Reverse Transcription Kit (Applied Biosystems). Reverse transcription of RNA for subsequent analysis was carried out at 25°C for 10 minutes, 48°C for 90 minutes, and 95°C for 5 minutes to achieve full-length cDNA transcription of alternatively spliced variants.

Amplification of hCAR SVs. For amplification of exon 1- and exon 9-containing hCAR SVs, specific primers were designed with PRIMER 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), and their sequence homology and specificity were checked by using BLASTn (http://www.ncbi.nlm.nih.gov). To amplify specifically hCAR SVs from human livers, nested polymerase chain reaction (PCR) was performed. First, hCAR SVs were amplified using 5′-GGAGAGGCTTCTCATACCCAG-3′ and 5′-TTCCTATCATCCGTTTATTCCAG-3′ primers in a total volume of 50 μl consisting of 1 μl of human liver cDNA reaction mixture, 10× PCR buffer, 1 mM MgCl₂, 10 pmol of each primer, 0.2 mM dNTP (Invitrogen), and 2.5 U of TaKaRa LA-Taq (TaKaRa Shuzo Co., Kyoto, Japan). The PCR condition was 32 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 90 seconds. cDNA yields of amplified SVs in some samples were low, so a second round of nested amplification was performed using the 5′-CAGGTGACATGCTGCCTAAG-3′ and 5′-TATCCAGGTGGTGTATCCAGT-3′ primers and appropriate dilutions of the first-round PCR products. The conditions used were the same as the first-round amplification except the number of cycles was 15 instead of 32 as in the first PCR.

Cloning and Cellular Expression of hCAR SVs. The amplified hCAR SVs were separated by 6% acrylamide gel electrophoresis. After gel staining, individual bands were excised, and PCR product was extracted using the Gel Extraction Kit for polyacrylamide gels (NucleoGen, Siheung-si, Korea). More than five different clones containing fragments from each band were cloned into a pGEM T-Easy Vector (Promega). Highly pure plasmid was obtained by means of the Plasmid Plus Midi Kit (Qiagen), amplified, and sequenced with T7 and SP6 (forward and reverse) universal primers and hCAR-specific primers. Sequencing was performed using the ABI Prism 3130XL Automated Sequencer (Applied Biosystems).

For expression of hCAR SVs, cloned hCAR fragments were transfected to expression vectors pCDNA 3.1 (Invitrogen) and pCMV Tag2B (BD Biosciences Clontech, Palo Alto, CA). The hCAR SVs in T-vector were digested with BamHI and HindIII, cloned into the expression vectors, and their sequences confirmed using the ABI Prisim 3130XL (Applied Biosystems). Four micrograms of each hCAR SV expression plasmid (WT, SV1–SV10) was transfected into COS-7 cells (1.0 × 10⁶ cells per well; Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfection, the cells were harvested and total protein was isolated as previously described. Twenty micrograms of total cellular proteins was separated by electrophoresis in a 12% SDS-PAGE gel (Invitrogen) and transfected onto Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). The membrane was incubated with polyclonal rabbit anti-human CAR antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a primary antibody and then incubated with goat anti-rabbit IgG antibody as a secondary antibody (Santa Cruz Biotechnology Inc.). The signals were assessed by densitometric analysis using LAS 3000 imaging system (FujiFilm, Tokyo, Japan).

Transactivation Activity Assay. Human hepatoblastoma (HepG2) cells (American Type Culture Collection, Rockville, MD) were plated in 12-well dishes at 3 × 10⁵ cells per well. Transfection experiments were performed 16 hours after cell seeding using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, the transfection mixtures contained 400 ng of CYP2B6 reporter construct [pGL2-basic luciferase reporter vector containing CYP2B6 promoter and phenobarbital-responsive enhancer module (PBREM)], pCDNA-CAR WT, pCDNA-CAR SVs expression plasmid, and 10 ng of internal control plasmid (pRL-TK vector (Promega) containing the herpes simplex virus thymidine kinase promoter to provide Renilla luciferase expression). The CYP2B6 reporter construct was produced as described previously (Wang et al., 2002).
et al., 2003). Twenty-four hours after transfection, cells were washed once with culture media and incubated for an additional 24 hours in media containing 10% charcoal/dextran-treated fetal bovine serum (HyClone Laboratories, Logan, UT) with or without 1 mM PB (Enzo Life Sciences, Farmingdale, NY). Twenty-four hours later, cells were harvested, lysed, and centrifuged at 2000g, and luciferase activity was determined according to manufacturer’s instructions (Luciferase Assay System; Promega) using an automated luminometer. Luciferase activity was normalized to total protein. All experimental values were averaged from triplicate determinations per experimental condition, and the experiments were conducted in triplicate.

Ligand-dependent induction of CYP2B6 promoter transactivation was also tested using various hCAR ligands (Fig. 5). After transfection, cells were washed once with media and then incubated in media containing 10% charcoal/dextran-treated fetal bovine serum (HyClone Laboratories) with PB (0.25 mM, 0.5 mM, or 1 mM) and CITCO (1 μM, 5 μM, or 10 μM), PHY (5 μM, 25 μM, or 50 μM), DEHP (1 μM, 5 μM, or 10 μM), or 0.1% DMSO (as a control) for 24 hours. Subsequently, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Quantitation of hCAR SVs mRNA in Human Livers. Quantification of hCAR variants mRNA was performed by specific quantitative real-time reverse-transcription PCR assays on an ABI PRISM 7900HT system (Applied Biosystems) as described previously (Jinno et al., 2004). The specific primers were designed for the detection of either the insertion or deletion of introns and exons as follows: a forward primer for hCAR WT, SV2, and SV4 was designed to span the exon 6/7 junction site; a forward primer for SV9 and SV10 was designed to span the exon 6/8 junction site and the intron 6/exon 7 junction site; a reverse primer for SV2 and SV4 was designed to span the intron 7/exon 8 junction site; a reverse primer for SV9 was designed to span the exon 8/9 junction site; and a reverse primer for SV10 was designed to span the intron 7/exon 8 junction site. The different hCAR splicing variants (or splicing events) were discriminated by these sets of primers, as well as the primer sequences, are depicted in Fig. 1 and Table 2. For normalization of quantity of initial mRNA, the percent levels of WT and SVs were calculated. From the relative variants value, the expression of each variant relative to WT was also calculated. For this, WT and SV mRNA levels were determined, and the expression of each variant because they had premature termination codons. SV6, with an exon 2 retention of intron 7 in SVs are indicated as a black box. The structure of each SV is depicted in Fig. 1 and Table 2. For normalization of quantity of initial mRNA, the percent levels of WT and SVs were calculated.

Immunoblot Analysis. Liver homogenates were centrifuged at 600g for 10 minutes at 4°C to precipitate the nuclear fraction. The supernatant was stored at -80°C. Protein concentrations of the nuclear fraction were quantitated using Bio-Rad Protein Assay Solution (BioRad, Hercules, CA). Thirty micrograms of nuclear proteins was separated on a 12% SDS-PAGE (Invitrogen) and transferred to a polyvinylidene difluoride membrane (GE Healthcare). The membrane was treated with rabbit anti-human CAR (1:200; Santa Cruz Biotechnology) and peroxidase-conjugated anti-rabbit goat IgG (1:4000; Santa Cruz Biotechnology) and peroxidase-conjugated anti-goat mouse IgG (1:4000; Santa Cruz Biotechnology). Chemiluminescence was determined using the Western blotting detection reagent, ECL-Plus (GE Healthcare). The protein band-images were read with a LAS 3000 imaging system and analyzed with Multi Gauge V 2.1 software (FujiFilm).

Statistical Analysis. Differences in hCAR SV quantities between samples of Korean and Caucasian livers were compared using the Wilcoxon rank sum test. The correlation between hepatic CAR and CYP2B6 protein expressions were tested using the Pearson correlation analysis in the SAS version 9.1.3 software (SAS Institute, Cary, NC). All other calculations were performed with the Statistical Package for Social Sciences (SPSS for Windows, release 11.0; SPSS, Chicago, IL).

**Results**

Eighteen hCAR SVs Including Four Novel SVs Were Identified in Human Liver mRNA. To identify novel SVs of hCAR, we amplified hCAR cDNA using primer pairs specific for exon 1 and exon 9. Reverse-transcription polymerase chain reaction was performed with the total RNA extract of individual human liver samples, and the PCR products were analyzed by acrylamide gel electrophoresis (Fig. 2A). Many different hCAR transcripts were amplified from pooled cDNA originating from 30 different Korean subjects with multiple hCAR SVs present in human livers (Fig. 2B). Cloning and sequencing of each SV revealed at least 18 different SVs (Fig. 3). The observed splicing events were as follows: complete deletion of exon 2 (140 bp), exon 3 (131 bp), exon 4 (170 bp), or exon 7 (117 bp); partial deletion of some exon (154 bp); and partial retention of intron 6 (95 bp) or 7 (198 bp). Each of the splicing events (five deletions and two insertions) occurred in multiple combinations, generating 18 SVs of hCAR. These different SVs of hCAR were named SV1-SV18, of which four SVs (SV1, SV2, SV3, and SV6) were identified for the first time in the present study. All these SVs contain intron 7 retention.

Several hCAR SVs were unlikely to encode functional proteins because they had premature termination codons. SV6, with an exon 2 retention of intron 7.

<table>
<thead>
<tr>
<th>Primer Sequences</th>
<th>Analyzed SVs</th>
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<tbody>
<tr>
<td><strong>Forward</strong></td>
<td>WT, SV5, SV8</td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
<td>SV2, SV4, SV7</td>
</tr>
<tr>
<td><strong>SV9</strong></td>
<td>Forward</td>
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<tr>
<td><strong>SV10</strong></td>
<td>Forward</td>
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**TABLE 2**

Primer sequences used for quantitation of human constitutive androstane receptor (CAR) splicing variants (SVs) in Fig. 1.
deletion, is out of the normal reading frame and introduced a premature termination codon. SV9, SV11, SV13, and SV15, derived from the use of the cryptic splice site in exon 2, have a deletion 67 bp from exon 2. SV10 has exon 2 and exon 4 deletions and also introduces a premature termination codon. In addition, SV13-18 had a deletion of part of the DNA binding domain, part of the hinge region, and a total or partial deletion of the ligand binding domain (LBD) region. According to Lamba et al. (2004), splicing variants with premature termination codons would more likely be rapidly degraded by nonsense-mediated decay.

Other SVs are expected to encode unique hCAR proteins. Deletion of exon 2 results in an in-frame loss of the 66 amino acids in the hCAR DNA binding domain. Exon 9 is 258 bp long and encodes 42 amino acids (the TGA stop codon is at nucleotide 128 in exon 9). Some SVs skipping the first 154 nucleotides of exon 9 may result in the loss of the carboxy-terminal 42 amino acids. However, this splicing event produces a new transcript and may use a cryptic AG splice site in exon 9, which causes a frame shift and encodes a unique carboxy-terminus with 29 amino acids instead of 154 amino acids.

hCAR SVs Differentially Transactivate CYP2B6 Promoter in Transfected HepG2 Cells. Transcriptional activation of the CYP2B6 gene is mediated by the PBREM present in the CYP2B6 promoter region (Wang et al., 2003). The hCAR SVs were functionally characterized in terms of ligand-dependent transactivation of PBREM.

To determine whether SVs were transcriptionally active like WTs, a reporter gene assay was performed using a cotransfection assay of CYP2B6 reporter construct with PBREM for each SV expression construct in the presence or absence of PB, a potent hCAR ligand. WT hCAR, when expressed in an immortalized cell line such as HepG2 cells, has been shown to translocate spontaneously to the nucleus (Zelko et al., 2001) and transactivate the PBREM reporter genes in the absence of any exogenous ligands (Sugatani et al., 2001; Jinno et al., 2004). Therefore, we first tested the transactivation activity of each SV in the absence of PB. Transfection of WT and SV2 significantly increased CYP2B6 PBREM-Luc activities (P < 0.05) compared with an empty vector (Fig. 4). Interestingly, the extent of transactivation by SV2 was significantly higher than that of WT in the absence of PB. Cotransfection of SV4 and SV5 also transactivated the CYP2B6 reporter gene to an extent comparable to WT in the absence of PB. Cotransfection of the other seven SVs failed to transactivate the CYP2B6 reporter gene regardless of PB treatment. Next, we determined whether SVs have altered responsibility to PB. We found that PB enhanced the WT- and SV2-mediated transactivation of CYP2B6 (~1.5-fold, P < 0.05 and ~1.7-fold, P < 0.05, respectively) at concentrations of 1 mM PB. The increase of transactivation activity by PB treatment was relatively low for SV4 and SV5. To rule out the possibility that the loss of transactivation activity of several SVs was due to the defective expression of hCAR proteins, immunoblotting was carried out to measure the wild-type and splice variant hCAR proteins in hCAR-transfected cells. The results showed high expression of expected sizes of either WT or SVs protein in the transfected cells. SV6, SV9, and SV10 could not produce recombinant proteins, probably because of the premature termination of translation.

Fig. 2. Identification of CAR SVs found in human livers. (A) Location of primers used for amplification of CAR SVs. Primary PCR and nested PCR were performed using a pair of F1 and R1 primers and a pair of F2 and R2 primers, respectively. Translation start site and termination site are indicated. (B) Separation of CAR SVs in acrylamide gel. PCR products from nested PCR were separated through acrylamide gel electrophoresis. Arrow indicates the wild-type CAR transcript. Locations of major abundant SVs with larger size and minor small SVs are indicated with SV names as in Fig. 3. DBD, DNA binding domain.

Fig. 3. Schematic representation of CAR SVs. All the exons are numbered. Shaded boxes represent the insertions and gaps represent the deletions. * start codon; ¥ stop codon. The predicted size of the amplified product and the predicted molecular weight of the transcript to make a full-length protein are on the right side. DBD, DNA binding domain; MW, molecular weight.

PTC = Premature termination codon
Similar splicing events were also found in major SVs in these regions are thought to contribute to the production of major hCAR gene in Caucasian livers because the SVs with splicing events reported the variability of splicing events in introns 6 and 7 of the CAR gene. As anticipated, SV9 showed no transactivation activity concentration of PHY slightly increased the transactivation activity could not significantly activate WT and SV2 CAR. Only the highest activation activity of WT. The same treatment with SV2-cotransfected a pretermination codon, it was used as the negative control. As shown since SV9 is devoid of exon 7 in the middle of the LBD and has known to activate certain SV forms of CAR (DeKeyser et al., 2011). Since CYP2B6 expression is regulated by CAR, the correlation between CYP2B6 and CAR WT expression and between CYP2B6 and total CAR expression, no statistically significant correlation was detected this, we designed specific primers to detect the partial or total retention of introns 6 and 7 (see Fig. 1) to detect the relative amount of each SV in 30 Korean livers and 20 Caucasian livers. The specificity of the primer pairs for each splice variant was tested by using SV clones as a template (unpublished data). First, we measured the total amount of hCAR transcript using exon 1-, exon 5-, and exon 9-specific primer sets, which are commonly present in all SVs. The amount of individual hCAR-WT (or SV5 or SV8), SV2, SV4 (or SV7), SV9, and SV10 mRNA was measured by real-time PCR using a known copy number of SV clones as a standard in 30 human Korean liver samples. Total amounts of hCAR transcripts in 30 human livers show a 73-fold difference (Fig. 6). These wide variations were similarly observed when primer sets for exon 1, exon 5, and exon 9 were used (unpublished data).

Next, we measured the relative amount of each SV among total hCAR transcripts in both Korean and Caucasian livers (Fig. 7). The most predominant SVs were SV4 (or SV7) and SV9 in Korean livers. The relative amount of WT (or SV5 or SV8) in Korean livers was only 4.5–16.0% of the total hCAR transcripts observed, whereas those of SV4 (or SV7) and SV9 were 34.5–57.6% and 25.1–34.4%, respectively. We also measured the relative quantity of each SV in 20 Caucasian livers. The relative amounts of the most abundant SVs, SV4 (or SV7) and WT (or SV5 or SV8), were 47.2–82.6% and 6.6–23.8%, respectively. The amount of SV9 was 6.0–22.3% in Caucasian livers. These results showed a wide range of expression variation of each SV in both ethnic groups. Through direct comparison of the relative proportion of each SV in terms of intron 6 and intron 7 retentions between Korean livers and Caucasian livers, interethnic difference of medians were tested by Wilcoxon rank-sum test. The median for all SVs showed significant differences between the two ethnic groups. Interestingly, the proportion of WT (or SV5 or SV8) and SV4 (or SV7), including functional hCAR transcripts, was much higher in Caucasian livers than in Korean livers. In contrast, the median amounts of SV2 with higher transactivation activity were 8.7% in Korean livers and 2.9% in Caucasian livers. In Korean livers, the expression of each SV was not significantly different among different genders or ages of the patients.

Protein Expression of hCAR Shows Interindividual Variation in Human Livers. Immunoblot analyses were conducted using the nuclear fractions of human liver samples to study whether various hCAR SVs were detectable as stable protein isoforms. The polyclonal antibodies recognizing the N-terminal regions shared by all the hCAR protein isoforms were used. Two or three distinct immunoreactive bands in the nuclear fractions of the human liver homogenates could be seen (Fig. 8). The upper band, 40 kDa, corresponded to the estimated size of the wild-type hCAR, and the broad band in the range of 32 to 35 kDa could be attributed to hCAR-SVs. Interestingly, despite heterogeneous hCAR transcripts, only three protein isoforms were found in Western blot analysis. The expression of different hCAR isoforms showed dramatic interindividual variation in their amounts and relative ratios. For example, whereas relative expression of hCAR WT was predominant in HL 8, HL10, HL13, and HL22, other samples, including HL9, showed darker lower bands, which indicates large amounts of hCAR SVs.

Since CYP2B6 expression is regulated by CAR, the correlation between CYP2B6 and CAR expression was investigated. The CYP2B6 expression shows also wide interindividual variation. However, between CYP2B6 and CAR WT expression and between CYP2B6 and total CAR expression, no statistically significant correlation was found (Fig. 8, B and C).
Discussion

Analysis of the human genome has revealed that up to 60% of genes express multiple mRNA isoforms via alternative promoter use or alternative splicing events, suggesting that it is one of the most significant components contributing to the functional diversity of proteins (Modrek et al., 2001). In the present study, we identified, by reverse-transcription polymerase chain reaction analysis, 18 hCAR SVs, including four novel SVs, which are generated as a result of alternative splicing of the hCAR pre-mRNA. Lamba et al. (2004) isolated at least 22 different hCAR SVs from Caucasian populations. Interestingly, only two SVs (SV7 and SV8 in the present study) are commonly described by our data and the data of Lamba and colleagues. These results suggest that a variety of SVs may be produced in other ethnic populations. Despite the large difference of hCAR SVs profiles between Koreans and Caucasians, it is noteworthy that the similarity was also apparent in terms of individual splicing event. For example, total deletion of exon 2, exon 9, or partial insertion of intron 6 or intron 7 are commonly observed in many SVs from both populations. Although Lamba et al. (2004) could not identify total insertion of intron 6 or intron 7, Jinno et al. (2004) observed those splicing events and even determined the relative frequency of splicing events in four Caucasian livers.

Among 18 hCAR SVs, 12 had deletion of exon 2, 11 had deletion of exon 9, 2 had insertion of both introns 6 and 7, and 6 had insertion of intron 7 in combination with other splicing events (Fig. 3). Deletion of exon 2 and exon 9 and insertion of total intron 6 produce truncated proteins at the C-terminus (e.g., hCAR SV1, SV3, SV6, and SV10). These proteins are expected to be nonfunctional because they lack helices H10/11 and H12 of the LBD, a key structure for interaction with coactivators and corepressors. H12 encodes the ligand-dependent activation domain, which is essential for ligand-dependent components contributing to the functional diversity of proteins (Modrek et al., 2001). In the present study, we identified, by reverse-transcription polymerase chain reaction analysis, 18 hCAR SVs, including four novel SVs, which are generated as a result of alternative splicing of the hCAR pre-mRNA. Lamba et al. (2004) isolated at least 22 different hCAR SVs from Caucasian populations. Interestingly, only two SVs (SV7 and SV8 in the present study) are commonly described by our data and the data of Lamba and colleagues. These results suggest that a variety of SVs may be produced in other ethnic populations. Despite the large difference of hCAR SVs profiles between Koreans and Caucasians, it is noteworthy that the similarity was also apparent in terms of individual splicing event. For example, total deletion of exon 2, exon 4, or exon 7 and partial insertion of intron 6 or intron 7 are commonly observed in many SVs from both populations. Although Lamba et al. (2005) could not identify total insertion of intron 6 or intron 7, Jinno et al. (2004) observed those splicing events and even determined the relative frequency of splicing events in four Caucasian livers.

Fig. 5. Effect of difference CAR ligands on transactivation activity of CAR SVs. CYP2B6 reporter vectors containing PBREM were transfected into HepG2 cells together with CAR SV expression vectors. Twelve hours later, transfected cells were treated for 24 hours with various concentrations of (A) PB (250 μM, 500 μM, or 1 mM), (B) CITCO (1 μM, 5 μM, or 10 μM), (C) PHY (5 μM, 25 μM, or 50 μM), or (D) DEHP (1 μM, 5 μM, or 10 μM). Luciferase activities were determined from three independent transfections using Dual Luciferase Reporter Assay System, and all experiments were done in triplicate. The data were displayed as the means ± S.E. of a single experiment representative of three independent experiments. Fold induction of CYP2B6 relative to DMSO (dimethylsulfoxide)-treated CTL was calculated. Data represent the mean ± S.E. of triplicate determination. *p < 0.05; **p < 0.01. P indicates the level of statistical difference from DMSO-treated group in each SV-transfected cells. CTL, control.

Fig. 6. Interindividual variation in total CAR mRNA transcript and its constituent SVs. cDNA synthesized from 30 ng of total RNA isolated from 30 independent human liver samples were subjected to TaqMan quantitative PCR. The copy numbers were obtained by comparing sample counts to a six-point standard curve for each primer/probe set included in the same plate. The results were normalized to the level of actin expression for each sample. As well as total CAR mRNA transcript, relative compositions of five different SVs groups (stacked graph) were determined by quantitative real-time PCR of the same liver samples.
transcriptional activation of nuclear receptors in general and also for the constitutive transactivation activity of hCAR (Dussault et al., 2002; Lamba et al., 2004).

In the present study, we identified for the first time SVs with retention of total intron 7. The SVs with this splicing event are SV1, SV2, SV3, and SV6. Among them, SV1 and SV3 produce truncated proteins as a result of the retention of total intron 6. SV6 also generates a very short truncated protein resulting from the deletion of exon 2. These three SVs were nonfunctional in transactivation assay, as anticipated (Fig. 4). The proteins of SV1 and SV3 were detected in the transfected cells, but they have no transactivation activity. SV6 protein was not detected in Western blot of the transfected cells, which may be due to the decreased protein stability of SV6 caused by premature termination; but SV2, with retention of intron 7, showed increased transactivation activity. The SV2 transactivated the CYP2B6 reporter gene approximately twice that of WT. The SV2 also has a premature termination codon in the middle of intron 7 inserted. Therefore, it does not contain the whole intact LBD structure that spans exon 4 to exon 9. Despite the premature termination in LBDs, SV2 showed higher responses to PB than any other SVs and WTs. The reason for this increase may be the structural changes caused by the insertion of new amino acids encoded into the intron 7 sequence so that SV2 may bind hCAR ligands more strongly or may translocate into the nucleus more effectively in a ligand-dependent manner. Besides WT and SV2, SV4 and SV5 showed transactivation activity when transfected into HepG2 cells. The common characteristics of functional hCAR SVs are the presence of intact exons in a row from exon 1 to exon 7 without any insertion or deletion of exons and introns. Partial insertion of intron 7 (15 bp, five amino acids) in SV4 did not affect significantly the ligand-independent or ligand-dependent transactivation activity. Also, partial deletion of exon 9 in SV5 also did not significantly change transactivation activity. These results suggest that, for transactivation activity of hCAR, an intact DNA binding domain is critical, and a considerable part of LBD encoded by exon 4 to exon 7 is necessary.

Next, we investigated the interindividual variations of hCAR transcripts in human livers, including the relative expression of each SV. First, we measured total hCAR transcript using primers specific for the amplification of common regions in all major SVs (exon 1, exon 5, and exon 9). The extent of interindividual variation was about 70-fold. Chang et al. (2003) also reported wide interindividual variation in hCAR mRNA expression, where a 240-fold difference was observed. Now it seems apparent that expression variation of hCAR mRNA is very large, and this variation may lead to functional variation in human livers. However, the profiling of hCAR SVs has been very limited by technical problems. For example, it is impossible to measure quantitatively all hCAR SVs at the same time in the same sample. Instead, specific primers were designed to detect abnormal junctions found in SVs, as in Fig. 1. Using these sets of primers, we can distinguish five different forms of splicing variants: WT, SV2, SV4, SV9, and SV10. As a result of the various combinations of alternative splicing events found in other regions, except intron 6 and intron 7, WT cannot be distinguished from SV5 or SV8.

### Table 1: Interindividual and interethnic differences in CAR SVs

<table>
<thead>
<tr>
<th>SV*</th>
<th>CAR SV / total CAR transcript (%)</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Korean (n=30)</td>
<td>Caucasian (n=20)</td>
</tr>
<tr>
<td>SV10</td>
<td>None</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>SV9</td>
<td>None</td>
<td>29.8 ± 2.4</td>
</tr>
<tr>
<td>SV2</td>
<td>High</td>
<td>9.7 ± 2.4</td>
</tr>
<tr>
<td>SV4+SV7</td>
<td>/None</td>
<td>45.1 ± 4.3</td>
</tr>
<tr>
<td>WT+SV5+SV8</td>
<td>/None</td>
<td>10.5 ± 3.1</td>
</tr>
</tbody>
</table>

**SV names in italics indicate loss-of-function SVs.**

**P** value is from Wilcoxon rank-sum test for difference of non-normally distributed median between ethnics.

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Fig. 7. Interindividual and interethnic differences in CAR SVs. Real-time PCR was performed for quantitation of each SV in 30 livers from Korean subjects and 20 livers from Caucasian subjects. The relative amounts of five different SVs groups (same as in Fig. 6) were calculated and compared between the ethnic groups as well as among individuals. The bars in the Korean samples are in the same order as in Fig. 6. Table below the graph shows mean, S.D., median, and range of relative amount of each SV in each ethnic group. **SV names in italics indicate loss-of-function SVs.**

**P** value is from Wilcoxon rank-sum test for difference of non-normally distributed median between ethnics.
SV4 also cannot be distinguished from SV7 by our method. Despite intrinsic technical problems, the relative quantities of nonfunctional SV9 and SV10 and highly functional SV2 could be determined in both Korean livers and Caucasian livers. The relative proportion of SV9 and SV10 in Koreans (29.8% ± 2.4% and 4.89% ± 1.0%, respectively, n = 30) were significantly different from those in Caucasian livers (12.8% ± 3.8% and 9.9% ± 3.3%, respectively, n = 20, P < 0.001). The sum of proportions of nonfunctional SV9 and SV10 was significantly higher in Koreans than in Caucasians (Fig. 7), but this does not mean that the total proportion of nonfunctional hCAR SVs in Caucasians is lower than in Koreans because we could not determine the exact quantities of the other nonfunctional hCAR SVs such as SV7, SV8, SV1, and SV3 from the present study. The range of SV4 + SV7 amounts in Caucasians (47.2–82.6%, median = 55.0%) is much higher than that in Koreans (45.5–76.6%, median = 45.6%). The amount of WT + SV5 + SV8 was also high in Caucasians compared with Koreans. Granting the problems in determining the exact proportions of functional hCAR SVs and nonfunctional hCAR SVs in each liver, it is noteworthy that significant differences in hCAR-SVs profiles are observed between Koreans and Caucasians. This difference may be due to the ethnic difference of genetic variations involved in splicing events. For example, a genetic variant of the CYP2D6 gene, CYP2D6*41, produces SV as a result of the genetic variation around splicing sites (Toscano et al., 2006). The frequency of this functional genetic variant is different among genetic variations in populations. This variation is expected to contribute to further complexity of hCAR-related functional variation in populations.

CYP2B6 expression is known to be upregulated by CAR. Therefore, it is expected that CYP2B6 expression is correlated with CAR expression. However, in the present study, we could not see a significant correlation between CYP2B6 expression and CAR expression in human livers. The possible reasons may include the genetic variation of CYP2B6 gene, limited numbers of human liver samples in our study, or complex expression of CAR SVs, although the exact mechanisms need to be further studied.

Here we evaluated interindividual and interethnic variations of hCAR-SVs profiles. From this profiling, it was found that the greater proportion of hCAR mRNA transcripts is nonfunctional, and the splicing events showed ethnic-dependent differences. The profiles of hCAR-SVs in each liver, it is noteworthy that significant differences in hCAR-SVs profiles are observed between Koreans and Caucasians. This difference may be due to the ethnic difference of genetic variations involved in splicing events. For example, a genetic variant of the CYP2D6 gene, CYP2D6*41, produces SV as a result of the genetic variation around splicing sites (Toscano et al., 2006). The frequency of this functional genetic variant is different among different ethnic groups, which leads to the ethnic difference of mean CYP2D6 SV amounts. To test this possibility, we cloned and sequenced regions covering exon 6-intron 6 and exon 7-intron 7; but genetic variations were not detected in any of the samples by full-length sequencing. These results suggest that the difference of SV profiles between these two ethnic groups is not due to the genetic variations of the hCAR gene. The mechanisms for this difference remain unknown.

Expressional variation of hCAR proteins was also investigated (Fig. 8). Despite the complex splicing events, only two to four major proteins were detected in immunoblot analysis. This might be due to the low stability of SV proteins or low resolution of polyacrylamide gel in this study because expected sizes of major forms of hCAR SVs are not easily distinguishable (27 kDa, 32 kDa, 34 kDa, 35 kDa, and 40 kDa). Granting this, dramatic individual variation in protein profiles was observed. Some livers showed higher expression of SVs, whereas others showed similar or much lower expression compared with WT. This variation is expected to contribute to further complexity of hCAR-related functional variation in populations.

**Authorship Contributions**

- **Participated in research design:** Lee, Shin, Choi.
- **Conducted experiments:** Choi, Jang, Cha.
- **Contributed new reagents or analytic tools:** Choi, Jang.
- **Performed data analysis:** Lee, Choi, Jang.
- **Wrote or contributed to the writing of the manuscript:** Lee, Choi, Jang, Shin.

**References**


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