Functional Characterization of Human UDP-Glucuronosyltransferase 1A9 Variant, D256N, Found in Japanese Cancer Patients

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ABSTRACT

SN-38 (7-ethyl-10-hydroxycamptothecin), an active metabolite of the antitumor prodrug irinotecan, is conjugated and detoxified to SN-38 10-O-β-D-glucuronide by hepatic UDP-glucuronosyltransferase (UGT) 1A1. Recent studies have revealed that other UGT1A isoforms, UGT1A7 and UGT1A9, also participate in SN-38 glucuronidation. Although several genetic polymorphisms are reported for UGT1A1 and UGT1A7 that affect SN-38 glucuronidation activities, no such polymorphisms have been identified for UGT1A9. In the present study, UGT1A9 exon 1 and its flanking regions were sequenced from 61 Japanese cancer patients who were all treated with irinotecan. A novel nonsynonymous single nucleotide polymorphism was identified in UGT1A9 exon 1, heterozygous 766G→A resulting in the amino acid substitution of D256N. The wild-type and D256N UGT1A9s were transiently expressed at similar protein levels in COS-1 cells, and their membrane fractions were characterized in vitro for the glucuronidation activities toward SN-38. The apparent K_m values were 19.3 and 44.4 μM, and the V_max values were 2.94 and 0.24 pmol/min/mg of membrane protein for the wild-type and D256N variant, respectively. The SN-38 glucuronidation efficiency (normalized V_max/K_m) of D256N was less than 5% that of wild-type UGT1A9. These results clearly indicate that the D256N variant is essentially nonfunctional with regard to SN-38 glucuronidation. These findings highlight the importance of further studies into the potential influence of UGT1A9 D256N variant to irinotecan metabolism in vivo.

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ABBREVIATIONS: SN-38, 7-ethyl-10-hydroxycamptothecin; UGT, UDP-glucuronosyltransferase; SN-38G, 7-ethyl-10-hydroxycamptothecin 10-O-β-D-glucuronide; PCR, polymerase chain reaction.
The 12-base pair partial *att* sequences at the 5′-end are underlined.

The base exchanged is underlined.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Primer Name</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>1st Amplification of exon 1s</td>
<td>UGT1A9-ZZF</td>
<td>5′-TTCTGGATCTGCTTCCATTTGCCATGATG-3′</td>
</tr>
<tr>
<td>from <em>UGT1A9</em> to <em>UGT1A7</em></td>
<td>UGT1A9-ZZR</td>
<td>5′-AACCAGGACCATCCATATAGCG-3′</td>
</tr>
<tr>
<td>2nd Amplification of <em>UGT1A9</em> exon 1</td>
<td>UGT1A9-1stF</td>
<td>5′-CCCAAGGGGAAAGCCATAGGC-3′</td>
</tr>
</tbody>
</table>

**Table 1**

Primer sequences used for sequencing exon 1 of the *UGT1A9* gene and plasmid construction

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Materials and Methods

**Materials.** SN-38 (lot 970507R) and SN-38G (lot 970507R) were kindly supplied by Yakult Honsha Co. Ltd. (Tokyo, Japan). Human liver polyA⁺ RNA was obtained from OriGene Technologies (Rockville, MD). COS-1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan).

**Human Genomic DNA Samples.** All of the 61 subjects in this study were Japanese cancer patients who were treated with irinotecan. The ethics committees of both the National Cancer Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating subjects.

**Polymerase Chain Reaction (PCR) Conditions for DNA Sequencing.** Exon 1 of *UGT1A9* was amplified from genomic DNA (150 ng) using 2.5 units of *Taq* (Takara, Kyoto, Japan) with 0.2 μM of the 1st amplification primers (Table 1). The first PCR conditions consisted of 30 cycles of 98°C for 5 s, 55°C for 5 s, and 72°C for 190 s. The PCR products were amplified by Ex-*Taq* (0.625 units; Takara) with the 2nd amplification primers (0.2 μM) designed to the intron sequences (Table 1). The second round of PCR was 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. This two-step PCR confers the specific amplification of the *UGT1A9* exon 1. These PCR products were then purified using a PCR product Pre-Sequencing kit (USB Co., Cleveland, OH) and were directly sequenced on both strands using an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with the primers shown in Table 1. The excess dye was removed by a DyeEx96 Kit (QIAGEN, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA analyzer (Applied Biosystems).

**Construction of Plasmids.** Oligo(dT) primed cDNA was synthesized from human liver polyA⁺ RNA using a SuperScript first-strand
synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cDNA encoding wild-type UGT1A9 was amplified from the single-stranded liver cDNA by the attB adaptor PCR and cloned into pDONR201 vector by the Gateway cloning technology (Invitrogen). Briefly, the UGT1A9 cDNA was first amplified by the 10-cycle PCR using the gene-specific primers containing the 12-base pair partial attB sequences at the 5’-end (Table 1). Then an aliquot of the reaction mixture was subjected to the second 25-cycle PCR using the attB adaptor primers (Table 1). The resulting attB-flanked product was cloned into pDONR201 vector using the Gateway BP reaction, involving a recombination between the attB site and the attP site. Mutations were introduced into the wild-type UGT1A9 cDNA clone in pDONR201, using a QuickChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) with the 5′-phosphorylated oligonucleotide primer (Table 1). To ensure that no errors had been introduced during the amplification process, all the plasmid constructs were verified by DNA sequencing of both strands. Subcloning of each UGT1A9 fragment from pDONR201 into pcDNA-DEST40 was performed by the Gateway LR reaction (a recombination between the attL site and the attR site).

Expression of Wild-Type and Variant UGT1A9s in COS-1 Cells. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The day before transfection, COS-1 cells were plated in 100-mm culture dishes at a density of 5.5 × 10⁴ cells/cm². On the following day, the culture medium was replaced with 8 ml of Opti-MEM (Invitrogen), and the expression plasmids were transfected using LipofectAMINE 2000 reagent (Invitrogen). The diluted DNA (14 µg in 810 µl of Opti-MEM) and the diluted LipofectAMINE 2000 reagent (48 µg in 810 µl of Opti-MEM) were combined and incubated for 20 min at room temperature. The resulting DNA-LipofectAMINE 2000 complex was directly added to each dish.

Forty-eight hours after transfection, the COS-1 cells were washed twice with ice-cold phosphate-buffered saline and harvested in 0.25 M sucrose-5 mM Hepes, pH 7.4 (buffered sucrose). The cell suspensions were sonicated three times with 10-s bursts using an ultrasonic processor USP-300 (Shimadzu, Kyoto, Japan), followed by centrifugation at 105,000g for 60 min at 4°C. The resulting pellets were resuspended in buffered-sucrose and stored at −80°C.

Western Blotting. Twenty micrograms of the membrane fraction proteins from COS-1 cells were resolved by SDS-polyacrylamide gel electrophoresis (10% gel) and electrophoretically transferred onto polyvinylidene difluoride membranes. Immunochromal detection of each protein was performed by chemiluminescence using rabbit anti-human UGT1A1 (diluted at 1:5000; BD Gentest, Woburn, MA) and donkey anti-rabbit Ig coupled to horseradish peroxidase (diluted at 1:2000). Chemiluminescence (ECL-plus; Amersham Biosciences, Piscataway, NJ) was detected and quantified using the Typhoon 9400 variable mode imager and ImageQuant analysis software (Amersham Biosciences). To confirm that the samples were evenly loaded, the blot was subsequently stripped in a stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min and reprobed with a polyclonal anticalnexin antibody (diluted at 1:10,000; Stressgen, San Diego, CA).

Enzyme Assay. The glucuronidation activities of wild-type and variant UGT1A9s were assayed as described previously (Hanioka et al., 2001a). Briefly, the incubation mixtures contained 50 mM Tris-HCl buffer, pH 7.4, SN-38 (final concentration, 2.5–150 µM; dissolved in dimethyl sulfoxide/0.05 N NaOH (1:1)), the membrane fraction of COS-1 cells (100 µg of protein), 10 mM MgCl₂, and 5 mM UDP-glucuronic acid. Because of the lack of any measurable effect on the enzyme activity, alamethicin was not employed in the assay. After preincubation at 37°C for 1 min, the reaction was started by the addition of UDP-glucuronic acid. The mixture was incubated at 37°C for 80 min, and the reaction was terminated with 100 µl of 10% (w/v) HClO₄. After centrifugation at 12,000g for 10 min at 4°C, the clear supernatant was filtered using a 0.45-µm polytetrafluoroethylene membrane filter and analyzed by high-performance liquid chromatography. Under the assay conditions employed in this study, SN-38 lactone and carboxylate forms are not distinguished, and hence, the SN-38 concentrations represent the combined total of lactone and carboxylate forms.

Data Analysis. Statistical comparisons were performed using Student's t test for unpaired data. Kinetic parameters were calculated using Prism 3.0 (GraphPad Software, Inc., San Diego, CA), using nonlinear regression of the Michaelis-Menten equation. The kinetic parameters were determined from three independent preparations of UGT1A9 protein.

Results

Genetic Variations in the Exon 1 of Human UGT1A9. Among the 61 Japanese cancer patients tested, a novel nonsynonymous single nucleotide polymorphism in exon 1 of the UGT1A9 gene was found in a patient as a heterozygote. As shown in the electropherograms (Fig. 2), the position of the variation was 766 (A of the translational start codon is 1) in exon 1, which was confirmed by repeating the PCR on genomic DNA and sequencing the newly generated PCR products. This genetic variant (766G>A) resulted in the amino acid substitution of D256N. To functionally characterize the nonsynonymous D256N variant, wild-type and variant UGT1A9s were expressed in COS-1 cells, and their SN-38 glucuronidation activities were determined.

Expression of Wild-Type and D256N Variant UGT1A9 in COS-1 Cells. Wild-type human UGT1A9 cDNA was successfully cloned from liver cDNA by the Gateway recombinational cloning method. The G to A substitution at 766 was introduced to the wild-type UGT1A9 cDNA by PCRBased site-directed mutagenesis. The wild-type and D256N variant were then transiently expressed in COS-1 cells. Figure 3A shows the representative Western blot of pooled samples from three independent transfections. For each sample preparation, relative UGT1A9 levels were determined using one of the wild-type membrane preparations as a standard. The blots were reprobed with a polyclonal anti-calnexin antibody to confirm that the samples were evenly loaded. As shown in Fig. 3B, a decrease in the relative expression level of D256N (0.76 ± 0.05) was slight but statistically significant by Student’s t test (p < 0.05). These relative expression levels were used for the normalization of the SN-38 glucuronidation activities of UGT1A9s described below.

Fig. 2. Electropherograms of UGT1A9 gene sequences at nucleotide 751 to 780 for wild-type (top) and heterozygous 766G>A (bottom) individuals. Arrows indicate the variant nucleotide positions.
SN-38 Glucuronidation Activities of the Wild-Type and D256N Variant UGT1A9s. Comparison of the functional properties of the wild-type and D256N variant UGT1A9s was made, while the SN-38 glucuronidation proceeded linearly within the incubation time (0.1 mg of protein/incubation and 80 min incubation time). The apparent enzyme kinetic parameters (K_m, V_max, and V_max/K_m) were estimated by fitting the initial velocity of enzymatic reaction, measured as a function of SN-38 concentration (2.5–150 μM), to the Michaelis-Menten equation. The representative nonlinear regression curves are depicted in Fig. 4.

Table 2 summarizes the apparent kinetic parameters for SN-38 glucuronidation by the wild-type and variant UGT1A9s. The wild-type UGT1A9 catalyzed SN-38 glucuronidation with an apparent K_m value of 19.3 μM, while that of the D256N variant was 44.4 μM. Their V_max values were 2.94 and 0.24 pmol/min/mg of membrane protein for the wild-type and D256N variant, respectively. When the V_max values were normalized taking the difference in their expression levels into account, the SN-38 glucuronidation efficiency ratio (normalized V_max/K_m) were 153 and 7.1 nM/min/mg of protein for the wild-type and D256N variant, respectively. These kinetic parameters clearly demonstrate that the D256N variant is nearly inactive toward SN-38 glucuronidation compared with the wild-type UGT1A9.

Discussion

It is increasingly apparent that an individual susceptibility to adverse drug reaction can be attributed to genetic factors. In this regard, polymorphism of UGT1A1 in irinotecan toxicity is one of the most well documented examples (Pirmohamed and Park, 2001; Tukey et al., 2002). Additional TA repeat in the A(TA)6TAA sequence of the UGT1A1 promoter (UGT1A1*28) has been shown to result in 30% to 80% reduction in transcriptional activity compared with wild-type UGT1A1 (Bosma et al., 1995; Beutler et al., 1998). The decrease in the level of UGT1A1 protein and, hence, glucuronidation activity results in an aberrantly high metabolic ratio of SN-38 to SN-38G. Recent pharmacogenetic studies (Ando et al., 2000; Iyer et al., 2002) have shown a positive correlation between the UGT1A1 promoter genotype and the severe toxicity of irinotecan, including diarrhea and leukopenia. To date, more than thirty polymorphic variations have been reported in the coding region of the UGT1A1 gene (Tukey and Strassburg, 2000). Some of these variants such as G71R and P229Q are also known to influence the SN-38 glucuronidation kinetics (Gagné et al., 2002; Jinno et al., 2003). Besides UGT1A1, recent kinetic studies in vitro have revealed that UGT1A7 and UGT1A9 participate in the glucuronidation of SN-38 (Ciotti et al., 1999; Hanioka et al., 2001b; Gagné et al., 2002). Guillemette et al. (2000) have identified three variant UGT1A7 alleles, UGT1A7*2 (N129K/R131K), UGT1A7*3 (N129K/R131K/W208R), and UGT1A7*4 (W208R). Among these variants, UGT1A7*3 and UGT1A7*4 have been shown to exhibit decreased SN-38 glucuronidation activity (20%–40% of the wild-type allele UGT1A7*1) (Gagné et al., 2002). However, the clinical importance of UGT1A7 polymorphism in irinotecan toxicity remains to be determined because of the rather limited tissue distribution of UGT1A7 in orlaryngeal and gastric tissues (Strassburg et al., 1997; Zheng et al., 2001; Vogel et al., 2001). In this regard, no significant association has been found between the presence of UGT1A7 variant alleles and the occurrence of
severe toxicity of irinotecan (Ando et al., 2002). In contrast to UGT1A7, UGT1A9 has been shown to be expressed in both liver and extrahepatic tissues such as colon and kidney (Strassburg et al., 1998; McQuirk et al., 1998). Therefore, polymorphism in the UGT1A9 gene could potentially influence the pharmacokinetics of irinotecan/SN-38 and irinotecan-induced toxicity, as addressed by Gagné et al. (2002).

Taking these previous studies into account, we have determined the nucleotide sequence of exon 1 of the UGT1A9 gene from cancer patients treated with irinotecan and identified a novel nonsynonymous variant 766G>A (D256N). Functional characterization revealed that the SN-38 glucuronidation efficiency (normalized $V_{\text{max}}/K_m$) of the D256N variant was less than 5% of wild-type UGT1A9. The apparent $K_m$ value of wild-type UGT1A9 (19.3 μM) in this study is nearly equal to that obtained using recombinant UGT1A9 expressed in insect cells (13.4 μM) (Hanioka et al., 2001b) and comparable with that of UGT1A1 expressed in COS-1 cells (11.5 μM) (Jinno et al., 2003). Nevertheless, Gagné et al. (2002) reported a $K_m$ value of 0.7 μM for the HEK293 cell-expressed UGT1A9. We have no explanation for this discrepancy at present, except for pointing to the difference in experimental conditions such as phosphatidylcholine and saccharolactone in the glucuronidation assay medium, which are absent in the present study.

The physiological significance of UGT1A9 polymorphism in irinotecan toxicity, in any case, mostly depends on the expression levels of UGT1A1 and UGT1A9 in the liver, although no quantitative information is available at present. A quite large interindividual variability has been reported for hepatic UGT1A1 level: more than a 30-fold variability was found both for UGT1A1 mRNA levels (Congiu et al., 2002) and estradiol 3-glucuronide level: more than a 30-fold variability was found both for UGT1A1 mRNA levels (Congiu et al., 2002) and estradiol 3-glucuronide level: more than a 30-fold variability was estimated by Ritter et al. (1999). These findings highlight the importance of carrying out further investigations into the possible influence of the UGT1A9 D256N variant with regard to irinotecan metabolism in vivo.

In conclusion, we identified a novel genetic variant of UGT1A9 in Japanese cancer patients; 766G>A results in the amino acid substitution of D256N, which is essentially non-functional for SN-38 glucuronidation. These findings highlight the importance of carrying out further investigations into the possible influence of the UGT1A9 D256N variant with regard to irinotecan metabolism in vivo.

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References


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