Novel Functional Polymorphisms in the UGT1A7 and UGT1A9 Glucuronidating Enzymes in Caucasian and African-American Subjects and Their Impact on the Metabolism of 7-Ethyl-10-hydroxycamptothecin and Flavopiridol Anticancer Drugs

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ABSTRACT
In vitro metabolic studies revealed that along with UDP-glucuronosyltransferase (UGT) 1A1, the hepatic UGT1A9 and the extrahepatic UGT1A7 are involved in the biotransformation of the active and toxic metabolite of irinotecan, 7-ethyl-10-hydroxycamptothecin (SN-38). Variant UGT1A1 and UGT1A7 alleles have been reported but the polymorphic nature of the UGT1A9 gene has not been revealed yet. To further clarify the molecular determinants of irinotecan-induced toxicity, we have identified and characterized the functionality of novel UGT1A9 polymorphisms and determined whether additional missense polymorphisms exist in UGT1A7. Using direct DNA sequencing, four single nucleotide polymorphisms (SNPs) were identified in the first exons of UGT1A7 and UGT1A9. One of the two amino acid substitutions found in the UGT1A9 gene, UGT1A9*3 (M33T), results in a dramatic decrease in SN-38 glucuronide formation, with 3.8% of the activity of the UGT1A9*1 allele. In turn, the glucuronidation of flavopiridol, an anticancer drug biotransformed predominantly by UGT1A9, remains unaffected, indicating a substrate-dependent impact of this variant. UGT1A9*3 is detected only in Caucasians and 4.4% of the population tested was found heterozygous (*1/*3). Two additional UGT1A7 SNPs were found exclusively in African-American subjects and generate five alleles (UGT1A7*5 to *9) when combined to the four known SNPs present in UGT1A7*2, *3, and *4. Upon functional analysis with SN-38, five out of nine UGT1A7 allozymes exhibited much lower SN-38 glucuronidation activities compared with UGT1A7*1, all having in common the mutational changes at codons 115 or 208. Results suggest that these low SN-38 glucuronidating alleles may represent additional molecular determinants of irinotecan-induced toxicity and warrant further investigations.

7-Ethyl-10-hydroxycamptothecin (SN-38) is the pharmacologically active metabolite of the anticancer drug irinotecan used globally in the first line treatment of advanced metastatic colorectal cancer (Douillard et al., 2000; Saltz et al., 2000). A major drawback of irinotecan-based chemotherapy is the high incidence of severe gastrointestinal and hematological toxicities (Gupta et al., 1994; Saltz et al., 1996; Cunningham et al., 1998; Rougier et al., 1998). Irinotecan-induced toxicities thus became a major concern in the treatment of cancer patients (Ledermann et al., 2001; Sargent et al., 2001). Glucuronidation by UDP-glucuronosyltransferase (UGT) enzymes is the main metabolizing reaction that contributes to SN-38 biotransformation in humans, yielding the inactive β-glucuronide form (SN-38G) (Gupta et al., 1994; Iyer et al., 1998). It is believed that SN-38G is deconjugated into SN-38 by intestinal glucuronidase enzymes and further causes diarrhea by direct enteric injury.
UGT1A1 was the first human UGT demonstrated to participate in the glucuronidation of SN-38 (Iyer et al., 1999). A strong correlation was observed between the UGT1A1 promoter polymorphism and rates of SN-38 glucuronidation, suggesting a main contribution of UGT1A1 to the in vivo biotransformation of SN-38 (Iyer et al., 1999). Two independent clinical studies further demonstrated a significant correlation between the polymorphic dinucleotide TA repeat (TA)_n of the UGT1A1 promoter, which affects the transcriptional level of the gene, and the occurrence of severe toxicities associated with irinotecan-based chemotherapy (Ando et al., 2000; Iyer et al., 2002). These findings indicate that glucuronidation of SN-38 would protect against irinotecan-induced gastrointestinal and hematological toxicities. However, the predictability of the UGT1A1*28 for irinotecan-induced toxicity was not complete, suggesting that the analysis of UGT1A1*28 would exclude a significant percentage of the population who experience severe toxicity. To further clarify the molecular determinants of irinotecan-induced toxicity, we previously performed a series of in vitro metabolic studies to identify UGTs relevant to SN-38 glucuronidation (Gagne et al., 2002). Of all 16 UGTs tested, UGT1A1, UGT1A7 and UGT1A9 showed the highest SN-38 glucuronidation capacities, with similar intrinsic clearance values (V_{max}/K_{m}) (Gagne et al., 2002). UGT1A9 presented a 2- to 10-fold greater affinity toward SN-38 compared with UGT1A1 and UGT1A7. UGT1A7 is not expressed in the liver and the gastrointestinal (GI) tract, and its role in the determination of systemic SN-38 concentrations remains unclear (Ciotti et al., 1999; Gagne et al., 2002). On the other hand, UGT1A1 and UGT1A9 are highly expressed in the liver, the primary organ involved in the detoxification of irinotecan, and also in the GI tract where the toxicity occurs (Congiu et al., 2002). Based on the finding that UGT1A9 participates significantly in the glucuronidation of SN-38 in vitro and according to its expression pattern, it would thus be relevant to search for polymorphic variation in this gene to further investigate the molecular determinants of irinotecan-induced toxicity.

The primary objective of the study was to examine the genomic sequences of the UGT1A9 gene, for which functional polymorphisms have not been described yet, as well as whether additional missense mutations exist in the UGT1A7 gene. The aims were to look for missense polymorphisms, to develop methods for single nucleotide polymorphism (SNP) detection, and to evaluate their functional properties after in vitro expression of enzyme variants. Previous attempts to find genetic polymorphisms in the human UGT1A9 gene in German subjects have been unsuccessful (Vogel et al., 2001). In turn, UGT1A7 is a polymorphic gene for which there are at present four known allelic variants (Guillemette et al., 2000; Zheng et al., 2001). Based on in vitro metabolic studies, the UGT1A7*3 and *4 variants may potentially lead to a poor SN-38 glucuronidating activity (Gagne et al., 2002). In the present study, three additional UGT1A7 genotypes with a low SN-38 glucuronidating activity were identified in addition to a UGT1A9 variant with barely detectable SN-38 glucuronidating activity. Given that the coinheritance of polymorphisms in UGT1A1, UGT1A7, and UGT1A9 may further impact patient’s susceptibility to irinotecan-induced toxicity, we have also elucidated the haplotype structure in subjects having the UGT1A9*3 polymorphism. Results suggest that further analysis of these variants to determine their impact on irinotecan metabolism in a clinical setting is warranted.

**Materials and Methods**

**Materials.** SN-38 was kindly provided by Dr. James Patrick McGovern (Pharmacia and Upjohn, Inc., Kalamazoo, MI) and dissolved in dimethyl sulfoxide. Flavopiridol was kindly provided by Dr. Robert J. Schultz (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD). SN-38G was produced from enzymatic assays with human liver microsomes, isolated, and quantified on an SN-38 calibration curve. All other chemicals and reagents used were of the highest grade and commercially available.

**DNA Samples.** Unrelated Caucasian subjects recruited at the Massachusetts General Hospital (Guillemette et al., 2000) and genomic DNA from African-American subjects used in a previous study were kindly provided by Robert Millikan (Guillemette et al., 2000). DNA samples of 201 Caucasian subjects were obtained from the Quebec Family Study (Lanouette et al., 2001). These samples had been anonymized before their reception in our laboratory. All subjects provided written consent for the use of their DNA for experimental purposes, and the present study was reviewed and approved by Institutional Review Boards (CHUL Research Center and Laval University, Quebec, Canada).

**Resequencing of the UGT1A9 Gene and Genotyping.** PCR was used to amplify the first exon of the UGT1A7 and UGT1A9 genes. Three pairs of primers were designed to amplify overlapping fragments covering the first exons, a small portion of the 5′-flanking region and the intron-exon boundary (Table 1). Specificity of primers was confirmed by direct sequencing of all PCR products. PCR amplification and DNA sequencing were performed according to protocols by Faucher et al. (2002). Amplicons were sequenced with an ABI 3700 automated sequencer using Big Dye (PerkinElmer Life Sciences, Boston, MA) dye primer chemistry. Samples were sequenced on both strands with nested primers listed in Table 1. Samples with ambiguous sequencing chromatograms and samples with SNPs were subjected to a second, independent amplification, followed by DNA sequencing. Sequences were analyzed with Staden preGap4 and Gap4 programs. These programs align sequence chromatograms and identify areas in which polymorphisms might be present. Each chromatogram was then evaluated individually to confirm variation in the sequences.

**UGT1A7 Haplotype Determination.** To discriminate the polymorphisms at codons 129/131 and 139, a PCR technique using TaqMan technology was used (Applied Biosystems, Branchburg, NJ). To discriminate the two alleles at codons 129/131, the exon 1 containing the codon 129/131 was amplified using primers 387 and 388, as described previously (Zheng et al., 2001). Two probes were designed to identify the two different alleles, the probe for N129/R131 allele was marked with FAM fluorochrome and the probe for K129/K131 allele was marked with TET fluorochrome. Also, specific primers were designed to amplify region of exon 1 containing codon 139. Specific 21-mer probes were designed to identify the two different alleles. One of the probes, E139-FAM, was homologous to the wild-type allele. The other probe, D139-VIC, contained the polymorphic nucleotide at codon 139 to be homologous to the D139 mutant allele. Each PCR reaction was performed with 25 ng of genomic DNA in a volume of 10 µl and containing 5 pmol of each primer and probe and 1× TaqMan universal PCR master mix. PCR conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The ABI prism 7000 system detected the different genotypes (Fig. 1, a and c). The polymorphism at codon 208 of UGT1A7 was genotyped by PCR-restriction fragment length polymorphism. The polymorphism at codon 208 creates a restriction site for RsaI enzyme. Digestion was
## Primer Sequences

<table>
<thead>
<tr>
<th>PRIMERS UGT1A9 SEQUENCES</th>
<th>PRIMERS UGT1A7 SEQUENCES</th>
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<td>Taqman analysis (codon 115)</td>
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<td>#18 5'-cgctggacggcaccattg</td>
</tr>
<tr>
<td>#38 5'-catccaat</td>
<td>#123 5'-ccctaagagaagtctgggg</td>
</tr>
<tr>
<td>#39 5'-gttgctgtagagatcatact</td>
<td>#124 5'-ccctaagagaagtctgggg</td>
</tr>
<tr>
<td>#41 5'-gttgctgtagagatcatact</td>
<td>#125 5'-ccctaagagaagtctgggg</td>
</tr>
<tr>
<td>#42 5'-gttgctgtagagatcatact</td>
<td>#126 5'-ccctaagagaagtctgggg</td>
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</tbody>
</table>

- **PCR amplification**
  - #37 5'-gttgctggtatttctccc
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  - #39 5'-gttgctgtagagatcatact
  - #41 5'-gttgctgtagagatcatact
  - #42 5'-gttgctgtagagatcatact

- **Taqman analysis (codon 115)**
  - #18 5'-cgctggacggcaccattg
  - #123 5'-ccctaagagaagtctgggg
  - #124 5'-ccctaagagaagtctgggg
  - #125 5'-ccctaagagaagtctgggg
  - #126 5'-ccctaagagaagtctgggg

- **Site-directed mutagenesis**
  - #544 5'-gttgctggtatttctccc
  - #545 5'-gttgctggtatttctccc
  - #546 5'-gttgctggtatttctccc
  - #547 5'-gttgctggtatttctccc
  - #548 5'-gttgctggtatttctccc

### SN-38 Glucuronidation by Novel Polymorphic UGT1A7 and UGT1A9 Allozymes

M33 5'-agtgccca

- **PCR amplification**
  - #18 5'-cgctggacggcaccattg
  - #123 5'-ccctaagagaagtctgggg

- **Site-directed mutagenesis**
  - E139-Fam 5'-atacttaaaggagagtttt
  - D139-Vic 5'-gttctctgatggctt

### Allelic specific oligonucleotides

- **N129/R131-FAM** 5'-ttaatgaccgaaaatt
- **Y3** 5'-atggctt
t

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- **Site-directed mutagenesis**
  - E139-Fam 5'-atacttaaaggagagtttt
  - D139-Vic 5'-gttctctgatggctt
was added every 2 days until colonies of resistant cells became visible and for amplification of geneticin-resistant cell populations.

Microsomes were prepared by differential centrifugation as described previously (Gagne et al., 2002). The crude cell extracts were centrifuged at 12,000 g at 4 °C for 22 min to remove nuclei and other cellular debris. Supernatants were centrifuged at 105,000g for 60 min at 4°C to obtain the membrane fraction, which was homogenized in the buffer described above. Protein concentrations were determined using the Bradford method according to the manufacturer's recommendations.

Fig. 1. Methods for SNPs detection. UGT1A7 and UGT1A9 first exons were amplified in unrelated subjects using specific primers for each gene. a, allelic discrimination PCR was used to genotype UGT1A7 codons 129/131. The probe marked with FAM fluorochrome was designed to detect the wild-type N¹²⁹/R¹³¹ allele. The other probe used to detect the polymorphic allele K¹²⁹/R¹³¹ was marked with TET fluorochrome. b, PCR products amplified with primers 17 and 18 were digested using RsaI enzyme to determine whether the patients were homozygous wild-type W²⁰⁸, heterozygous W²⁰⁸/R²⁰⁸, or homozygous R²⁰⁸. The 590-bp fragment represents the undigested PCR product, whereas the 336- and 264-bp fragments result from the digestion of the 590-bp amplicon. c, allelic discrimination PCR was used to genotype the novel polymorphism at codon 139 of the UGT1A7 gene. The FAM fluorochrome was used to mark the wild-type probe E¹³⁹, and the VIC fluorochrome was used for the polymorphic probe D¹³⁹. d, ASOs were designed to genotype the novel polymorphic variation at codon 115 of UGT1A7 gene after specific amplification of the corresponding gene confirmed by sequencing. e and f, a similar strategy was further used to detect variant at codons 3 and 33 of the UGT1A9 gene. Duplicate filters were hybridized separately with the corresponding γ-²P-labeled oligonucleotides. The positive signals detected with both ASOs indicated heterozygous individuals for the polymorphism in contrast with a positive signal with one probe only, which indicated that the subject was homozygous. Random samples with mutations were sequenced and the presence of SNPs confirmed.
To determine the level of UGT proteins expressed in the microsomal fractions obtained from the stably transfected cells, Western blot analyses were conducted as follows. Microsomal proteins (10 μg) from HEK293 cells stably expressing human UGT1A9 and UGT1A7 variants were separated by 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto nitrocellulose membranes and probed with the anti-human UGT1A1 antiseraum RCT71 (1:1,000 dilution) specific for the conserved C-terminal region of the protein (Gagne et al., 2002). To normalize sample loading, blots were reprobed with anti-calnexin antibody (1:2,000 dilution; StressGen Biotechnologies, Victoria, BC, Canada), to detect a second endoplasmic reticulum-resident protein. A donkey anti-rabbit IgG antibody conjugated with the horseradish peroxidase (Amersham Biosciences, Oakville, ON, Canada) was used as the secondary antibody (1:10,000 dilution). The resulting immunocomplexes were visualized using an enhanced chemiluminesence kit (Renaissance, QC, Canada) and exposed on Kodak XB-1 film. The relative levels of UGT1A allozymes and calnexin were determined by integrated optical density using Bioimage programs visage 110S (Genomic Solution Inc., Ann Arbor, MI) and compared with the *1 respective alleles.

**Enzyme Assays.** Recombinant allozymes were assayed for UGT activity with the two anticancer agents, SN-38 and flavopiridol, as substrates. Microsomal fractions from HEK293 (40–60 μg) were added to a reaction mixture (100 /H9262 l) containing 50 mM Tris-HCl, pH 7.3, 10 mM MgCl2, 100 μg/ml phosphatidylcholine, and 2 mM UDP-glucuronic acid. SN-38 (lactone) was added in concentrations ranging from 0.1 to 200 μM, whereas flavopiridol was used at two concentrations: 5 and 200 μM. Commercially available human liver microsomes (Human Cell Culture Center Inc., Laurel, MD) were incubated under the same conditions for all experiments. Time-course experiments were performed to determine the linearity of the glucuronidation reaction. Due to the lack of apparent enzyme latency, inclusion of detergent was found to be unnecessary for assessment of the full glucuronidating potential of UGT-expressing HEK cell membranes. For the determination of Vmax and Km, HEK293 cells stably expressing UGT1A9 enzymes were incubated in the presence of 10 concentrations of SN-38 ranging from 0.1 to 200 μM and incubated for 30 min as described above, whereas UGT1A7 microsomes were incubated for 3 h. All reaction rates were shown to be linear in these conditions. Kinetic parameters for novel alleles of UGT1A7 were not determined because of analytical sensitivity. Reactions with SN-38 were stopped by the addition of 200 μl of MeOH + 1% HCl (2 N), followed by centrifugation at 14,000g for 10 min. The supernatants were filtered through a 0.22-μm membrane and 100 μl of water was added to the filtrate. For the detection of SN-38 and its glucuronide (SN-38G), 10-μl samples were injected in a liquid chromatographic system (HPLC) coupled to a fluorescence detector (see below).

An HPLC method was developed to quantify the rates of SN-38 glucuronidation from the various microsomal fractions under study. The HPLC system used was an Alliance 2695 (Waters, Milford, MA) equipped with a 50 × 3.2 mm Colboms C18 column (Phenomenex, Torrance, CA). The chromatographic separation was achieved with a two-solvent gradient system: solvent A (water + 1 mM ammonium formate) and solvent B (MeOH + 1 mM ammonium formate). A linear gradient starting at 20% solvent B was generated over a 3-min period and at a constant flow rate (0.7 ml/min) until a plateau was reached at 65% solvent B and held for another 0.8 min. A gradient ranging from 65 to 95% solvent B was generated during the following 2 min. Finally, the column was reequilibrated at 20% solvent B for 2 min. The column was connected to a fluorescence detector model 474 (Waters), and the molecules were excited at a wavelength of 370 nm and an emission of 425 nm. The retention times for SN-38 and SN-38G were 4.49 and 3.12 min, respectively. Because we could not perform kinetic analysis with the UGT1A9*3 using the previously used electrospray ion-trap mass spectrometry method (Gagne et al., 2002), the fluorescence detection was preferred because it was more sensitive in these conditions and allowed the detection of SN-38G formed by UGT1A9*3 microsomes at low concentrations of SN-38. The Km values calculated for the human liver microsomes using both analytical methods were shown to be similar (6.8 ± 0.3 μM with the LCQ detector; Gagne et al., 2002) and 4.8 ± 0.8 μM with the fluorescent detection (data not shown). Glucuronidation assay using flavopiridol as substrate were performed as described previously (Ramirez et al., 2002). Relative glucuronidation activities for flavopiridol (five and seven glucuronides) were determined for 1 h using 5 and 200 μM of substrate and in the same experimental conditions as used for SN-38.

**Immunofluorescence Visualization.** One cSNP found in the UGT1A9 first exon was located in the signal peptide; thus, immunofluorescence experiments were designed to localize the expressed protein within the cells. Stable HEK293 cells expressing human UGT1A9*1, UGT1A9*2, and UGT1A9*3 and also with cells transfected with pcDNA3 vector alone were seeded on culture slides (VWR Scientific, West Chester, PA) and allowed to grow for 18 h. Then, cells were washed three times with PBS and fixed for 20 min with paraformaldehyde 2% (w/v) (Sigma-Aldrich, St. Louis, MO) in PBS. The slides were washed three times for 40 min in PBS containing saponin 0.2% (w/v) (Sigma-Aldrich). After three washes with PBS, the cells were incubated for 30 min with 0.2% gelatin in PBS (w/v) (Sigma-Aldrich). The permeabilized cells were incubated with a rabbit anti-UGT1A9 primary antibody (RC-71) at a 1:1,000 dilution (v/v) in PBS containing 0.1% saponin and 1.5% bovine serum albumin. Slides were incubated for 1 h and then washed three times with PBS. A goat anti-rabbit secondary antibody (Alexa Fluor 488; Molecular Probes, Eugene, OR) was added at a 1:4,000 dilution in the same buffer as the primary antibody, and slides were incubated for 30 min at room temperature in the dark. Cells were then washed three times with PBS. Cell counterstaining was achieved by incubating the slides for 30 s in the dark at room temperature with a 1:1,000 (v/v) dilution of 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Finally, cells were washed with PBS and mounted with a mounting medium (Sigma-Aldrich). For visualization, a Fluoview confocal microscope (BX-61; Olympus, Melville, NY) with a 100× objective was used.

**Statistical Analysis.** Results were expressed as mean ± S.D. Differences in kinetic parameters between UGT allelic variants were evaluated for statistical significance by paired Student’s t test. All tests were two-sided. The haplotype frequencies were estimated using the Phase 0.1 software and Hardy-Weinberg equilibrium, and linkage disequilibrium analyses were performed using Arlequin 2.0 software (Schneider et al., 2000; Stephens et al., 2001).

**Results**

**Identification of Two Novel Missense Mutations in the Human UGT1A9 Gene and Their Distribution in Healthy Individuals.** The strategy used to identify polymorphisms in the UGT1A9 gene was a PCR amplification of the exon 1, followed by direct DNA sequencing. Inclusion of a portion the adjacent intron and 5'-flanking region in the PCR fragment was performed to ensure the specific amplification of the UGT1A9 gene. The UGT1A9 was ressequenced on both strands for 35 subjects. DNA samples from Caucasian-American subjects was shown to contain one SNP, whereas an additional SNP was observed in an African-American subject. No insertion-deletion events were observed within the area sequenced.

The nucleotide change producing one of the cSNP found in Caucasians was a change of a G to an A at nucleotide 8. The polymorphic change results in a cysteine into a tyrosine (C8Y) in the signal peptide of the UGT1A9 protein corresponding to the UGT1A9*2 allele. The second nucleotide change, T98C, leads to a methionine to a threonine at codon 98.
33 (M33T) corresponding to the UGT1A9*3 allele. Figure 2, a and b, illustrate the sequence analysis of three genotypes: homozygous wild type *1/*1 and heterozygous *1/*2 or *1/*3.

To determine the allelic frequency of UGT1A9 allosymes in the population, we genotyped unrelated subjects, including 301 Caucasians of whom 201 were French-Canadians, and 20 African-American subjects. Only one African-American individual had the C3Y mutation (UGT1A9*2), whereas 12 individuals, all Caucasian subjects, were shown to have the M33T mutation (UGT1A9*3) (Fig. 1f). A total of 4.4% of individuals were found heterozygous for the UGT1A9*3 M33T allele in the French-Canadian population and 3% of the remaining Caucasian-American subjects. None of the 20 African-American subjects were found to have the UGT1A9*3 allele (M33T) (Table 2).

**Identification of Two Novel Polymorphisms in the Coding Region of the UGT1A7 Gene and Haplotypic Structure Analysis of the UGT1A7 Gene.** The exon 1 of UGT1A7 was amplified by PCR in 117 subjects, 54 Caucasians, and 63 African-Americans and then sequenced. Two novel polymorphisms were found at codon 115 and 139 (Fig. 2, c and d). At codon 115, a nucleotide change of a G to an A leads to an amino acid change from glycine to serine (G115S). A G-to-C mutation at codon 139 leads to an amino acid change from glutamate to aspartate (E139D).

When combined with the previously described variations at
SN-38 Glucuronidation by Novel Polymorphic UGT1A7 and UGT1A9 Allozymes

Functional change

Amino acid change Cys<sup>3</sup> Met<sup>33</sup> "Wild type" Tyr<sup>3</sup> Met<sup>33</sup> Similar activity for SN-38 and flavopiridol Cys<sup>3</sup>Thr<sup>33</sup> Decreased activity for SN-38; similar activity for flavopiridol

Population characteristics

<table>
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<tr>
<th>Allele Frequency</th>
<th>Cys&lt;sup&gt;3&lt;/sup&gt; Met&lt;sup&gt;33&lt;/sup&gt;</th>
<th>Tyr&lt;sup&gt;3&lt;/sup&gt; Met&lt;sup&gt;33&lt;/sup&gt;</th>
<th>Cys&lt;sup&gt;3&lt;/sup&gt;Thr&lt;sup&gt;33&lt;/sup&gt;</th>
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TABLE 2

Allelic frequency and prevalence of UGT1A9 alleles

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</tr>
<tr>
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<tr>
<td>*1/*2</td>
</tr>
<tr>
<td>*1/*3</td>
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</table>

* Subjects homozygous for variant UGT1A9 alleles were not observed in the population tested.

TABLE 3

Allelic frequency and prevalence of UGT1A7 alleles

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<tr>
<td>*2</td>
</tr>
<tr>
<td>*3</td>
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* Subjects homozygous for variant UGT1A9 alleles were not observed in the population tested.

nine variant alleles were separated in two categories: the common and the rare alleles. The common alleles *1, *2 and *3, are present at an allelic frequency of 0.31 to 0.32. The rare alleles are UGT1A7*4 to *9, with frequencies between 0.002 and 0.025. The allelic frequencies for the polymorphisms at codon 115 and 139 were 0.04 and 0.06, respectively and found specifically in African-American individuals.

Recombinant Allozyme Western Blot Analysis. Semi-quantitative Western blot analyses (Fig. 3, a and b) showed high levels of immunoreactive UGT protein in all microsomal fractions from HEK293 cell lines stably expressing UGTs. An anti-calnexin polyclonal antibody was also used in combination as an internal reference. Significant expression of all UGT1A7 and UGT1A9 alleles was found adequate, allowing enzymatic assays to be performed.

Recombinant UGT1A7 and UGT1A9 Enzyme SN-38 Kinetics. The functional genomic studies were focused on two anticancer drugs, SN-38 and flavopiridol. UGT1A7 was previously shown to have the highest intrinsic clearance with SN-38 as substrate along with UGT1A1 and UGT1A9 (Ciotti et al., 1999; Gagne et al., 2002), whereas UGT1A9 is the main UGT involved in the metabolism of flavopiridol (Hagenauer et al., 2001; Ramirez et al., 2002).

Our results thus demonstrate that the M<sup>33T</sup> polymorphism dramatically impairs the conjugation rate of SN-38, whereas no significant effect was observed with the UGT1A9*2 allozyme. The formation of SN-38G by the UGT1A9*3 enzyme is markedly reduced, with only 3.8% residual activity compared with the wild-type enzyme using 5 μM SN-38. In contrast, the formation of flavopiridol-G was not statistically different for UGT1A9*2 and UGT1A9*3 compared with the UGT1A9*1 allele at both low and high concentrations (5 and 200 μM of flavopiridol) (data not shown), suggesting a substrate-specific impact of this amino acid variation in the UGT1A9 protein.

To determine whether the amino acid change at codon 33 affects enzyme activity by an alteration of kinetic properties, glucuronidating activity of UGT1A9 allozymes was assessed using a wide range of SN-38 concentrations (0.1–200 μM). A nonsignificant higher apparent K<sub>m</sub> value for the UGT1A9*2 variant was observed as determined at least in three independent experiments. Both UGT1A9*1 and UGT1A9*3 alleles demonstrated similar apparent K<sub>m</sub> values of 3.0 ± 0.5 and 3.2 ± 1.0, respectively (Table 4). As a result, decreases in level of enzyme activity observed for the UGT1A9*3 allele could not be attributed to the alterations of substrate affinity. However V<sub>max</sub> values were about 27 times lower for
UGT1A9*3 compared with UGT1A9*1 (11.9 ± 2.6 versus 316.3 ± 52.0 pmol/min/mg of protein; p < 0.002).

In the analysis of UGT1A7 allozymes, the highest SN-38 glucuronidating activity was observed for UGT1A7*1, *2, *6, and *7 (Table 3). Three novel low activity alleles were identified and the *5, *8, and *9 alleles presented 38 to 76% lower rates of SN-38G formation compared with UGT1A7*1, similar to the range of activity of the *3 and *4 alleles previously identified as low SN-38 glucuronidating activity alleles (Gagne et al., 2002).

Subcellular Localization of UGT1A9 Allozymes. To determine whether the subcellular localization of UGT1A9 was affected by the codon 3 mutational polymorphism in the signal peptide region, immunofluorescence experiments were carried out. Coloration with DAPI was restricted to the nucleus (Fig. 4, e, h, and k), whereas the low background ob-
TABLE 4
Kinetic parameters for SN-38 glucuronidation by human UGT1A9 allozymes

<table>
<thead>
<tr>
<th>UGT1A9</th>
<th>Apparent K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Catalystic Efficiencies</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A9*1</td>
<td>3.0 ± 0.5</td>
<td>316.3 ± 52.0</td>
<td>105</td>
<td>316.3 ± 52.0</td>
</tr>
<tr>
<td>UGT1A9*2</td>
<td>5.1 ± 1.8</td>
<td>324.4 ± 95.1</td>
<td>63</td>
<td>324.4 ± 95.1</td>
</tr>
<tr>
<td>UGT1A9*3</td>
<td>3.2 ± 0.9</td>
<td>11.9 ± 2.6</td>
<td>4</td>
<td>11.9 ± 2.6</td>
</tr>
</tbody>
</table>

* p < 0.002 compared with UGT1A9*1.

Table 4: Kinetic parameters for SN-38 glucuronidation by human UGT1A9 allozymes.

Discussion

UGT1A1 was initially identified as the main UGT enzyme involved in the glucuronidation of SN-38 into SN-38G in humans (Iyer et al., 1998). However, additional studies revealed that the hepatic UGT1A9 and the extrahepatic UGT1A7 contribute significantly to the formation of SN-38G in vivo (Ciotti et al., 1999; Hanioka et al., 2001; Gagne et al., 2002). The present study focused on the findings and the functional characterization of novel UGT1A7 and UGT1A9 polymorphic variants. We also performed the analysis of the co-occurrence of SNPs in the UGT1A1, UGT1A7, and UGT1A9 genes associated with altered formation of SN-38G in vitro, given that they are clustered in a 200-kilobase genomic region. After resequencing the first exons of UGT1A7 and UGT1A9 genes, four polymorphic sites in the targeted regions were identified. Two polymorphic UGT1A9 variants were discovered, UGT1A9*2 C<sup>Y</sup> and UGT1A9*3 M<sup>M</sup>T<sup>P</sup>. In addition, the presence of two novel nonsynonymous UGT1A7 SNPs, G<sup>1</sup>S and E<sup>139</sup>D, combined with previously described missense polymorphisms at codons 129/151 and 208, generated five additional UGT1A7 alleles (*5 through *9). Based on the in vitro functional genomics assays, the UGT1A7*3, *4, *5, *8, and *9 alleles and the UGT1A9*3 allele were all identified as low SN-38 glucuronidating alleles. Given that the biotransformation of SN-38 by UGT1A1 was recently shown to protect against side effects induced by irinotecan therapy in cancer patients (Iyer et al., 1998, 2002; Ando et al., 2000), our findings suggest that polymorphic variations associated with low SN-38G formation in the UGT1A7 and UGT1A9 genes, alone or combined with UGT1A1, potentially lead to an altered clearance of SN-38 in vivo.

Previous work by our group has identified several missense polymorphisms in the human UGT1A7 gene (Guillemette et al., 2000; Woolley et al., 2000; Guillemette, 2003), and their association with an increased risk of cancer was further demonstrated (Vogel et al., 2001; Zheng et al., 2001; Strassburg et al., 2002). Four UGT1A7 alleles have been described and were subsequently found in independent populations (Zheng et al., 2001). Combined with the two novel SNPs identified at codons 115 and 139, there are at least nine polymorphic alleles of the UGT1A7 gene. In the population studied, the frequencies of the novel UGT1A7*5 to *9 alleles were comparable to that of the previously described UGT1A7*4 allele and significantly lower compared with the frequencies of the UGT1A7*1, *2, and *3 common alleles. Also, a differential ethnic distribution of these novel SNPs was evident. The UGT1A7*4 <sup>G115S</sup>E<sup>139D</sup> and the UGT1A9 <sup>C</sup>Y variant alleles are all restricted to African subjects and are not observed in the relatively large number of Caucasian subjects (n = 301) included in our study. Inversely, the UGT1A9*3 (<sup>M</sup><sup>33T</sup>) allele is observed only in the Caucasian population but not in the African-Americans, although a large population of African-Americans should be screened to validate this observation and before conclusion of its genetic distribution may be made. Overall, these findings indicate a clear interethnic variation in the distribution of UGT1A7 and UGT1A9 polymorphic alleles. In a previous article by Vogel et al. (2001), German individuals were sequenced and no UGT1A9 variants were detected. In turn, a recent publication reports a SNP in the coding region of the UGT1A9 gene (D256N) found in a Japanese patient and which significantly alters the glucuronidation of SN-38 in vivo (Jinno et al., 2003). Again, these results support the idea of evolutionary differences in the distribution of UGT polymorphisms.

The methionine at codon 33 of UGT1A9 is located in a portion of the protein highly conserved among members of the UGT1A subfamily and particularly those sharing the highest homology of sequence with UGT1A9, such as UGT1A7, UGT1A8 and UGT1A10 (Fig. 5). This suggests that a nonconservative amino acid change in this portion of the transferase could result in modified enzymatic properties. In vitro functional data demonstrate a dramatic decrease in the catalytic activity of the UGT1A9 protein when the methionine is replaced by a threonine. The residual activity of this allele was barely detectable with 27 times less formation of SN-38 than the UGT1A9*1 allele, whereas the T<sup>33</sup> variation did not impair the affinity (K<sub>m</sub>) of the transferase for SN-38. On the other hand, immunofluorescence experiments showed that the UGT1A9*2 C<sup>Y</sup> variant leads to an adequate targeting of the transferase to the endoplasmic reticulum, despite a mutation in the leader peptide.

Even though the UGT1A9*3 allele was not found to be
widely distributed in the Caucasian population tested (frequency of 3 to 4.4%), it remains that this polymorphism could lead to a low SN-38 glucuronidator phenotype. Besides, a recent study by Kurkela et al. (2003) suggests that the UGT1A9 protein, when expressed in baculovirus, is capable of homodimerization. Several studies support the intermolecular association of transferases and suggest that UGTs may function as homodimers and oligomers with significant glucuronidation activities (Peters et al., 1984; Gschaidmeier and Bock, 1994; Meech and Mackenzie, 1997; Ishii et al., 2001). Furthermore, Meech and Mackenzie (1997) showed that the transferase activity of rat UGT isoforms was abolished by a leucine-to-arginine substitution in a highly conserved microregion among UGTs, only few amino acids from the UGT1A9 codon 33 polymorphism identified in this study (Peters et al., 1984; Gschaidmeier and Bock, 1994; Meech and Mackenzie, 1997; Ishii et al., 2001). Thus, if UGT1A9 proteins interact to form catalytically active dimers as suggested by the study by Kurkela et al. (2003), the UGT1A9*3 polymorphism would then produce deleterious effects on the glucuronidation of compounds catalyzed primarily by this enzyme, such as SN-38. In turn, the impact of this genetic variant is clearly

Fig. 4. Immunofluorescence localization of UGT1A9*1, UGT1A9*2, and UGT1A9*3 proteins. HEK293 cells stably expressing pcDNA3 (a) or human UGT1A9 alleles (d, g, and j) were fixed, permeabilized, and then treated with a rabbit anti-UGT1A primary antibody (RC-71), followed by a goat anti-rabbit secondary antibody. Cell counterstaining of the nuclei was performed using DAPI (b, e, h, and k). To confirm the localization of the UGT proteins, a combination of the images obtained with the antibodies and the counterstain are shown in c, f, i, and l.
substrate-specific because this mutational change had no effect on flavopiridol, an anticancer agent mainly glucuronidated by UGT1A9 (Hagenauer et al., 2001; Ramirez et al., 2002). In addition, a significantly increased activity of the UGT1A9*3 protein compared with the *1 protein was observed for few substrates of this isoenzyme (data not shown) (Guillemette and Villeneuve, 2003).

Several studies support the influence of the UGT1A1 promoter polymorphism (TA7) on SN-38 glucuronidation capacities in vivo (Iyer et al., 1998; Ando et al., 2000; Iyer et al., 2002). Two independent investigations revealed that a partial deficiency in the UGT1A1 SN-38 biotransforming enzyme increases the risk of severe neutropenia and diarrhea in cancer patients treated with irinotecan-based chemotherapy (Ando et al., 2000; Iyer et al., 2002). However, the predictability of the variant UGT1A1 promoter genotype for irinotecan-induced toxicity was not complete, suggesting that the analysis of the UGT1A1*28 allele would exclude a significant percentage of the population who experience severe toxicity due to an altered UGT metabolic pathway. Although a recent study of Japanese cancer patients treated with CPT-11 did not reveal the association between UGT1A7 genetic variants and toxicity events (Ando et al., 2002), any of these studies explored the influence of UGT1A9 genetic variants. It is of interest to mention that one-half of the subjects with the UGT1A9*3 allele did not carry the UGT1A1*28 allele. Consequently, if UGT1A9*3 allele is associated with altered SN-38 glucuronidation in vivo, which still remains to be demonstrated, our findings would lay emphasis on the necessity to analyze both genes to predict patients at higher risk for toxicity. The lack of association of the low UGT1A7 glucuronidating genotypes with irinotecan-associated toxicity is explained by the fact that UGT1A7 is not expressed in the liver and the GI tract and that its influence on the systemic SN-38 glucuronidation may not be sufficient to affect toxicity outcome compared with UGT1A1 and UGT1A9. Thus, the novel polymorphisms of the UGT1A7 found only in African-Americans subjects would be predicted to have limited impacts on the SN-38 metabolism in vivo. However, these polymorphisms may contribute to modify risk of cancer.

![Multiple protein sequence alignment of UGT1A proteins at selected positions. UGT1A7*1, UGT1A9*1, and their genetic variant proteins UGT1A7 (a) and UGT1A9 (b) are aligned with close members of the UGT1A subfamily and the rat UGT1A7 isoenzyme. The varying amino acid positions are indicated with bold characters.](image-url)
as found for the most common variant allele of the UGT1A7 gene, UGT1A7*3 (Guillemette, 2003).

In conclusion, findings suggest that the low SN-38 glucuronidating UGT1A9*3 allele may represent an additional molecular determinant of irinotecan-induced toxicity and warrant further investigations to determine its effect on the metabolism of irinotecan in vivo.

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References


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