Lack of Association between Common Polymorphisms in UGT1A9 and Gene Expression and Activity

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ABSTRACT:

Interindividual variability in the glucuronidation of xenobiotics metabolized by UDP-glucuronosyltransferase 1A9 (UGT1A9) suggests the presence of functional UGT1A9 variants. The aim of this study was to evaluate whether the putative functionality of the UGT1A9 variants −118T/rs, I399C>T, and −2152C>T variants were 0.39, 0.39, 0.02, and 0.02 in the livers, respectively. The I399C>T variant was in complete LD (r² = 1) with −118T/rs, (linked alleles: C and T, respectively). Complete LD between these two variants was also found in the HapMap samples (frequencies of −118T/rs, and I399C>T = 0.38), I399C>T and −118T/rs, correlated with neither UGT1A9 activities nor mRNA levels. Because of the low frequencies of the −2152C>T variants, an effect on phenotype could not be evaluated. Our data demonstrate that the common I399C>T and −118T/rs, polymorphisms do not explain interindividual variation in hepatic UGT1A9 activity and mRNA expression and are in complete LD in the donor liver samples we studied.

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J.R. and W.L. contributed equally to this work.

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ABBRVIATIONS: UGT, uridine diphosphate-glucuronosyltransferase; SN-38, 7-ethyl-10-hydroxycamptothecin; SNP, single nucleotide polymorphism; LD, linkage disequilibrium; CEPH, Centre d’Etude du Polymorphisme Humain; PCR, polymerase chain reaction; MPAG, mycophenolic acid phenyl glucuronide; UDPGA, uridine 5′-diphosphoglucuronic acid; FLAVO-7-G, flavopiridol-7-glucuronide; bp, base pair(s); KS, Komolgover-Snimoff.
rard et al., 2004). In particular, the linked −275A and −2152T alleles have been found to be associated with both higher UGT1A9 protein expression and increased rates of propofol and mycophenolic acid glucuronidation compared with individuals homozygous for the wild-type genotypes (Girard et al., 2004). However, the low allele frequencies of −275A (4–6%) and −2152T (3–6%) in Caucasians may not be sufficient to explain the variability in pharmacokinetic parameters observed with some pharmaceutical compounds metabolized by UGT1A9 (Girard et al., 2004; Innocenti et al., 2005).

The I399C→T variant was recently identified as a novel and common polymorphism in the immediate downstream intron of the UGT1A9 exon and was reported as being in low linkage disequilibrium (LD) (r² ≈ 0.19) with variants at positions −2152, −275, and −118 (Girard et al., 2006). This SNP was also significantly correlated with increased UGT1A9 protein levels and activities.

To evaluate whether the putative functionality of UGT1A9 variants −118T→C, I399C→T, −275T→A, and −2152C→T could be confirmed in an independent study, genotype-phenotype correlations were investigated in Caucasian human livers. A luciferase assay was additionally performed to further study the function of the −118T→C polymorphism. To confirm allelic frequencies and LD pattern, DNA samples from Caucasians belonging to the International HapMap Project were studied. Our results were then compared with previously published data regarding the functionality of UGT1A9 polymorphisms.

Materials and Methods

Study Populations. Samples (n = 106) from two cohorts of unrelated Caucasian individuals were studied, with approval of institutional review boards.

Cohort 1: Human Liver Samples. DNA, RNA, and human liver microsomes from 46 donors were processed through Dr. Mary Relling’s laboratory at St. Jude Children’s Research Hospital (Memphis, TN). The hepatic tissue was provided by the Liver Tissue Procurement and Distribution System (funded by Grant NO1-DK-9-2310) and by the Cooperative Human Tissue Network. For Cohort 2: CEPH Samples. Genotyping.

Cohort 1 had been previously genotyped for UGT1A9 (Girard et al., 2004; Innocenti et al., 2005). In particular, the linked −275A and −2152T alleles have been found to be associated with both higher UGT1A9 protein expression and increased rates of propofol and mycophenolic acid glucuronidation compared with individuals homozygous for the wild-type genotypes (Girard et al., 2004). However, the low allele frequencies of −275A (4–6%) and −2152T (3–6%) in Caucasians may not be sufficient to explain the variability in pharmacokinetic parameters observed with some pharmaceutical compounds metabolized by UGT1A9 (Girard et al., 2004; Innocenti et al., 2005).

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Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR reactions were performed using IQSYBR Green Supermix (Bio-Rad, Hercules, CA) and the UGT1A9 specific primers 5′-GAGGAACATTATATAAGCCACG-3′ (forward) and 5′-TGCCAAAGCATCAGCAATTGCT-3′ (reverse). 18S RNA was used as an internal control with primers 5′-GAGGTCTTCTTTTATAAGG-3′ (forward) and 5′-GATGACCGTATATGAGCACTCT-3′ (reverse). Reactions were performed in triplicate and included standard curves for both the target and control genes. Expressions of UGT1A9 and 18S RNA were calculated on the basis of the equations generated from their respective standard curves. The UGT1A9 mRNA levels were expressed as the ratio of UGT1A9 to the control gene, 18S, multiplied by a dilution factor of 200 as cDNA was diluted before quantification of 18S.

Construction of UGT1A9 Promoter Reporter Vectors. The UGT1A9 promoter region (−1 to −1040, according to the A in the translation initiation codon ATG) containing either −118T9 or −118T10 was subcloned into the pGL3-basic vector containing the firefly luciferase enzyme (Promega, Madison, WI). Briefly, a PCR was carried out with Proofstart DNA polymerase (Qiagen, Valencia, CA) to generate the amplicon from a −118T9/10 heterozygote DNA with primer sequences UGT1A9KPN (5′-GATCAGCGTACCGTGTCTTCTGCGAGGCCTTCT-3′) and UGT1A9HIND (5′-ATGAAATACCTCAGAGCATCGCTGAGACAAAC-3′). To facilitate the subcloning, the UGT1A9KPN and UGT1A9HIND primers were designed to contain a KpnI and a HindIII site, respectively. The modified nucleotides are underlined in the primer sequence listed above. After the modified products were digested, the 1040- or 1041-bp products were cloned into the KpnI/HindIII site of the pGL3-basic vector. All constructs were sequenced to exclude PCR errors and ensure the orientation of the construct before transfection.

Cell Culture and Transient Transfection. HepG2 and HEK-293 cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine and 10% fetal bovine serum in a humidified atmosphere (5% CO2-95% air) at 37°C. To compare the relative expression of the luciferase gene, pGL3UGT1A9uc*9 and pGL3UGT1A9uc*10 were transfected into the HepG2 and HEK-293 cell lines. The pGL3-basic vector was transfected into HEK-293 cell lines. Briefly, the cell lines were seeded into a 12-well plate 1 day before transfection. The cells were then cotransfected with 1 μg of pGL3UGT1A9uc*9 and 0.1 μg of pRL-TK plasmids (Promega) and combined with 2.5 μl of transfection medium (Promega) in 400 μl of serum-free medium (Invitrogen), according to the manufacturer’s instructions. The cells were incubated at 37°C under an atmosphere of 5% CO2 for 1 h. After incubation, the culture medium (2 ml) was added to the wells, and cells were incubated for 24 additional h. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega), following the manufacturer’s instructions. Relative luciferase activities were normalized to the activities of the internal control for Renilla luciferase activity. The transfections were performed in triplicate and repeated twice.

Statistics. The frequency distribution of the UGT1A9 mRNA levels (KS distance = 0.28, p = 0.009) and the MPAG formation rates (KS distance = 0.21, p = 0.04) were not normally distributed. They were log-transformed before statistical analyses, after which they passed the normality test (KS distance = 0.10, p > 0.10). Formation of FLAVO-7-G was apparently normal between intake of any of these UGT inducers and phenotypes (p > 0.05, univariate linear regression, data not shown). A significant correlation was also observed between the mRNA levels and the glucuronidation activities of both mycophenolic acid (r = 0.52, p < 0.001, n = 35) and flavopiridol (r = 0.43, p = 0.01, n = 35). Age and gender did not have a significant effect on UGT1A9 phenotypes (p > 0.05, univariate linear regression, data not shown). A history of alcohol and drug intake (phenobarbital/dexamethasone) was available for 28 donors; the smoking history of 39 individuals was known. No statistically significant associations were found between intake of any of these UGT inducers and phenotypes (p > 0.05, univariate linear regression; data not shown).

UGT1A9 Variants. The allele frequencies of the four variants were similar in cohorts 1 and 2 (Table 1). The −118T9/10 and I399C>T polymorphisms were present at high frequency, whereas the −275T>A and −2152C>T variants were relatively rare. All alleles were in Hardy-Weinberg equilibrium. In both cohorts, complete LD (r2 = 1.00) was observed between 1) −118T9/10 and I399C>T and 2) −2152T>A and −2152C>T. There was very low LD between −118 and −275 (r2 = 0.01 and 0.05 for cohorts 1 and 2, respectively). The haplotype structure and frequencies are presented in Table 2. The frequencies of haplotypes I and II were very similar in cohorts 1 and 2 (χ2 = 3.2, df = 2, p = 0.21).

Association between mRNA Levels, Activities, and UGT1A9 Polymorphisms. Correlations between genotypes and phenotypes were only tested with −118T9/10 and I399C>T because of the low frequency of the −275T>A and −2152C>T polymorphisms in our liver samples (2%). No significant differences were observed between the −118T9/10, −118T9/10 and −118I9/10 I399C>T and −2152C>T polymorphisms were in perfect LD, associations identical to those observed with −118T9/10 were found between UGT1A9 and I399C>T (Fig. 1).

Luciferase Reporter Gene Assay. Significant basal promoter activity of both T9 and T10 constructs was observed compared with the pGL3-basic empty vector (p < 0.01, data not shown). No significant
differences in luciferase activity were observed between the \(-118T_9\) and \(-118T_{10}\) alleles (\(p = 0.05\)).

**Discussion**

Although clinical studies show interindividual variability in the clearance of mycophenolic acid (Kuypers et al., 2005) and in the capacity to glucuronidate flavopiridol (Innocenti et al., 2000), we find no evidence that variability in \(UGT1A9\) expression or activity is related to previously identified common polymorphisms (Girard et al., 2006). We first focused on the high frequency variant \(-118T_9\).

The functionality of the \(-118T_9\) polymorphism had been suggested by a reporter assay that used a small promoter construct (\(-152\) to \(-171\) bp, according to the \(A\) in the translation initiation codon ATG) transfected into HepG2 cells (Yamanaka et al., 2004). As this construct included only the proximal promoter, we thought it might be insufficient to reflect the regulation of the promoter activity in vivo. To include the distal promoter, we constructed a longer subclone spanning more than 1 kilobase of the 5’-flanking sequence, a region that has been demonstrated to have full \(UGT1A9\) promoter activity (Gregory et al., 2003, 2004). By using two different cell lines, we observed similar promoter activity between the two alleles, suggesting that the promoter polymorphism does not alter function. This is in agreement with others studies reporting only a marginal fold elevation (1.35–1.43) of reporter expression by the \(T_{10}\) allele, compared with the \(T_9\) allele, using 154- and 170-bp constructs (Girard et al., 2006) and showing that \(T\) insertions/deletions in the \(UGT1A8, UGT1A9,\) and \(UGT1A10\) promoters do not affect gene transcription (Mackenzie et al., 2005).

For phenotypic assessment of \(UGT1A9\) activity, we used flavopiridol and mycophenolic acid. Flavopiridol has been shown to be a relatively specific substrate for \(UGT1A9\) in experiments using recombinant enzymes and propofol as enzyme inhibitor (Hagenauer et al., 2001; Ramirez et al., 2002). Even though we could not quantify the rate of formation of FLAVO-7-G using standards, we believe we can detect accurately differences in glucuronidation activities, as the activity measurements were done under linear assay conditions and using a substrate concentration equal to the \(K_m\) determined under the experimental conditions reported in this study. In addition, a significant and modest correlation was observed between the glucuronidation of flavopiridol and mycophenolic acid, a commonly used \(UGT1A9\) probe (Bernard and Guillemette, 2004; Girard et al., 2004; Miles et al., 2005). However, the lack of high correlation suggests that both drugs may be glucuronidated to a certain extent by other UGTs (Hagenauer et al., 2001; Ramirez et al., 2002; Bernard and Guillemette, 2004; Miles et al., 2005; Picard et al., 2005). Levels of correlations similar to those observed between the formation of

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**TABLE 2**

**UGT1A9 haplotype frequencies in three Caucasian cohorts**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>(-2152C&gt;T)</th>
<th>(-275T&gt;A)</th>
<th>(-118T_{9,10})</th>
<th>(I399C&gt;T)</th>
<th>Our Data</th>
<th>Girard et al. (2006): Cohort 3 (n = 84)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>C</td>
<td>T</td>
<td>9</td>
<td>C</td>
<td>54 (0.59)</td>
<td>65 (0.54)</td>
</tr>
<tr>
<td>II</td>
<td>C</td>
<td>T</td>
<td>10</td>
<td>T</td>
<td>36 (0.39)</td>
<td>46 (0.38)</td>
</tr>
<tr>
<td>III</td>
<td>C</td>
<td>T</td>
<td>9</td>
<td>T</td>
<td></td>
<td></td>
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<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>T</td>
<td>A</td>
<td>9</td>
<td>T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>T</td>
<td>A</td>
<td>9</td>
<td>C</td>
<td>2 (0.02)</td>
<td>9 (0.08)</td>
</tr>
</tbody>
</table>

*The number of samples in the haplotypes were predicted using the frequency data reported by Girard et al. (2006).

**TABLE 3**

**UGT1A9 mRNA levels and substrate activities across the \(-118T_{9,10}, -118T_{9,10},\) and \(-118T_{10,10}\) genotypes**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>(9/9)</th>
<th>(9/10)</th>
<th>(10/10)</th>
<th>(p^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Log (UGT1A9 mRNA/18S mRNA)})</td>
<td>2.48 ± 0.84</td>
<td>2.79 ± 0.93</td>
<td>1.88 ± 0.92</td>
<td>0.12</td>
</tr>
<tr>
<td>(\text{Log apparent MPAG formation (log 10 nmol/min/mg protein)})</td>
<td>0.38 ± 0.16</td>
<td>0.46 ± 0.21</td>
<td>0.29 ± 0.19</td>
<td>0.10</td>
</tr>
<tr>
<td>(\text{FLAVO-7-G formation)})</td>
<td>0.28 ± 0.10</td>
<td>0.34 ± 0.10</td>
<td>0.26 ± 0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*p determined by analysis of variance.

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**FIG. 1.** Comparison of \(UGT1A9\) mRNA levels (left) and glucuronidation activities of mycophenolic acid (center) and flavopiridol (right) between \(UGT1A9 I399\) genotypes. *p values for the comparison of phenotypes between \(CC\) and \(TT\) individuals are shown.
FLAVO-7-G and MPAG in our study (r = 0.62) were observed between the glucuronidation rates of propofol and MPAG (r = 0.62) in another study (Girard et al., 2004). The lack of association observed between -118T>C, and either the UGT1A9 mRNA level or activities is consistent with other studies (Girard et al., 2004; Innocenti et al., 2005). Overall, these results suggest that the -118 polymorphism is unlikely to regulate UGT1A9 expression.

We could not confirm in our livers either the low level of LD previously observed between I399C>T and the other three UGT1A9 variants (Girard et al., 2006), or the functionality of I399C>T. We found complete LD between I399 and -118 not only in our liver set but also in the HepMap CEU samples for which the I399 genotypes are publicly available. We could not confirm a previous report of higher glucuronidation rates of mycophenolic acid in individuals homozygous for TT compared with CC (Girard et al., 2006). To the best of our knowledge, that report did not control for multiple comparisons and might have detected a false-positive association.

As for the effect of the -2152C>T and -275T>A variants, we could not study their association with phenotypes because of their low frequency in our liver samples. Although heterozygous samples have been associated with higher UGT1A9 protein expression (Girard et al., 2006), 9% of the samples studied were of non-Caucasian ethnicities, which might have confounded the findings. In addition, as these SNPs are not common in Caucasians, their clinical effect might be difficult to assess in clinical trials that enroll subjects from predominantly Caucasian populations.

All variants in the present study were genotyped in a previous study of 42 Caucasians (Girard et al., 2006). This allows comparison of the allele and haplotype frequencies between the two studies. For simplicity, we will refer to this third sample set as cohort 3. Variant allele and haplotype frequencies are listed in Tables 1 and 2, respectively. We predicted three haplotypes for 106 samples (cohorts 1 and 2 combined). In contrast, six haplotypes were inferred for the 42 samples in Girard et al. (cohort 3). The haplotypes from cohort 3 were significantly different from those predicted for cohorts 1 (χ2 = 22.0, df = 5, p = 0.0005) and 2 (χ2 = 30.5, df = 5, p < 0.0001). A possible explanation for the difference in haplotype structure and LD pattern observed between the two studies might be population stratification in the samples used in cohort 3 (Girard et al., 2006). As our data are publicly available, we could not confirm a previous report of complete LD between I399 and -118 polymorphism (Girard et al., 2004). The lack of association observed not only in our liver set but also in the HepMap CEU samples for which the I399 genotypes are publicly available. We could not confirm a previous report of higher glucuronidation rates of mycophenolic acid in individuals homozygous for TT compared with CC (Girard et al., 2006). To the best of our knowledge, that report did not control for multiple comparisons and might have detected a false-positive association.

References

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