The UDP-Glucuronosyltransferase (UGT) 1A Polymorphism c.2042C>G (rs8330) Is Associated with Increased Human Liver Acetaminophen Glucuronidation, Increased UGT1A Exon 5a/5b Splice Variant mRNA Ratio, and Decreased Risk of Unintentional Acetaminophen-Induced Acute Liver Failure

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ABSTRACT

Acetaminophen is one of the most widely used nonprescription antipyretic and analgesic drugs worldwide. Although considered very safe when used at recommended dosages, excessive dosing of acetaminophen can lead to serious liver injury. Studies by the Acute Liver Failure Study Group have identified acetaminophen as the leading single cause of acute liver failure (ALF) in the United States (Larson et al., 2005). About half of the cases result from intentional acute overdose, most likely in an attempt to elicit serious self-injury; the remaining cases appear to be unintentional resulting from other causes or a race- or ethnicity-matched population. Together, these findings suggest that rs8330 is an important determinant of acetaminophen glucuronidation and could affect an individual’s risk for acetaminophen-induced liver injury.

Introduction

Acetaminophen is one of the most widely used nonprescription antipyretic and analgesic drugs worldwide. Although considered very safe when used at recommended dosages, excessive dosing of acetaminophen can lead to serious liver injury. Studies by the Acute Liver Failure Study Group have identified acetaminophen as the leading single cause of acute liver failure (ALF) in the United States (Larson et al., 2005). About half of the cases result from intentional acute overdose, most likely in an attempt to elicit serious self-injury; the remaining cases appear to be unintentional resulting from chronic administration of multiple doses.

The mechanisms underlying acetaminophen-induced hepatotoxicity have been studied extensively (Larson, 2007). At therapeutic doses, acetaminophen is cleared primarily by conjugative metabolism (glucuronidation and sulfation). However, after higher (toxic) doses, conjugative pathways are overwhelmed and increased metabolism by the alternate...
cytochrome P450 oxidative pathway results in significant formation of the toxic electrophilic metabolite N-acetyl-p-benzoquinoneimine (NAPQI). NAPQI is normally rapidly detoxified by conjugation with glutathione, but excessive NAPQI formation exhausts available glutathione supplies. Acetaminophen overdose is effectively treated by administration of the glutathione precursor N-acetylcysteine. In support of this mechanism, various risk factors can influence susceptibility to acetaminophen hepatotoxicity via effects on these metabolic pathways. For example, chronic alcohol use can enhance toxicity through increased CYP2E1–mediated NAPQI formation (Larson, 2007).

Although it has been speculated that genetic variability may predispose some individuals to an increased risk of acetaminophen-induced ALF (Patel et al., 1992; Court et al., 2001; Rauchschwalbe et al., 2004; Zhao and Pickering, 2011), to date, only one published study has addressed this hypothesis. In that report, the association of polymorphisms in genes encoding three different glutathione S-transferase (GST) enzymes was investigated in 104 patients who had overdosed on acetaminophen (Buchard et al., 2012). Although none of the GST genotypes evaluated was associated with serum alanine aminotransferase levels, a sensitive biomarker of hepatocellular injury, a borderline significant (P = 0.05) association was found between copy number variation of the GST-T1 gene and trough prothrombin time (an indicator of clinical outcome). However, the direction of the association (gene deletion resulting in a better predicted outcome) was the opposite of what would be expected if glutathione conjugation of toxic acetaminophen metabolites by the GST enzymes protected against liver injury. This finding was suggested by the authors to be the result of increased glutathione availability in individuals lacking the GST-T1 gene since a previous mouse model study showed resistance to acetaminophen hepatotoxicity with higher glutathione levels in mice lacking the GST-P1 gene (Henderson et al., 2000).

Hepatic glucuronidation is one of the principal mechanisms by which acetaminophen is detoxified and cleared from the human body. Studies in our laboratory and others have identified genetic polymorphisms that alter hepatic glucuronidation enzyme expression and function (Girard et al., 2004, 2005; Krishnaswamy et al., 2005b; Court, 2010). The primary aim of the current study was to use a well-established in vitro model of interindividual variability of drug glucuronidation to identify genetic polymorphisms associated with variable acetaminophen glucuronidation in human liver. We also established the most likely mechanism by which the identified polymorphisms influence drug glucuronidation and then determined the frequencies of these polymorphisms in patients who had developed ALF as a consequence of acetaminophen use. Our results indicate that a common single nucleotide polymorphism (SNP) in the UDP-glucuronosyltransferase 1A (UGT1A) gene (rs8330) is associated with increased hepatic acetaminophen glucuronidation, possibly through effects on UGT1A gene splicing. Furthermore, this putative protective gene variant appears to be present at a lower frequency in patients who had unintentionally developed acetaminophen-induced ALF compared with a population with a similar racial or ethnic background.

Materials and Methods

Reagents. UDP-glucuronic acid (sodium salt), alamethicin, acetaminophen, and acetaminophen glucuronide were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were of analytical or better grade. Oligonucleotide primers used for sequencing and real-time polymerase chain reaction (PCR) assay were synthesized by the Tufts Core Facility (Tufts University, Boston, MA).

Human Liver Tissues. Liver samples from 48 donors with no known liver disease were obtained from either the National Disease Research Interchange (Philadelphia, PA) or the Liver Tissue Procurement and Distribution Service (Minneapolis, MN) with the approval of the Tufts University Institutional Review Board. All livers were either intended for transplantation but had failed to tissue match or were normal tissue adjacent to surgical biopsies. Donors were self-identified non-Hispanic whites and included 37 male subjects and 11 female subjects with a mean age of 43 years (range 2–75 years). Smoking history was positive for 16 donors, and significant alcohol use (defined as 14 or more drinks per week) was positive for 11 donors. Complete details of donor demographics, including available medication history for individual livers, have been reported elsewhere (Court, 2010).

Microsome Preparation. Human liver microsomes were prepared by differential ultracentrifugation as previously described (Court et al., 1997). Microsomal pellets were suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 20% glycerol and kept at −80°C until use. The protein concentration of human liver microsome samples was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard. The quality of the liver samples was ascertained by reference to at least 10 other glucuronidation activities measured in this laboratory using the same set of livers. Livers that consistently showed low activity values (≥2-fold lower for all measured activities) relative to the median activity value for the entire liver set were excluded from study.

Acetaminophen Glucuronidation Activities. The rates of in vitro glucuronidation were determined by high-performance liquid chromatography for all liver microsome samples using the method we have described previously (Court et al., 2001) at three different acetaminophen concentrations (0.1, 2, and 40 mM) with a fixed UDP-glucuronic acid concentration (20 mM). Activities were measured in duplicate, and results were averaged.

DNA from Acute Liver Failure Patients. DNA samples were obtained with appropriate consent from patients enrolled by the Acute Liver Failure Study Group, a consortium of U.S. liver centers established in 1998 to define more completely the causes and outcomes of ALF (Olapowicz et al., 2002). Standard entry criteria for acute liver failure were used. All patients were considered to have had an acute hepatic injury of less than 28 weeks' duration and demonstrated an international normalized ratio of ≥1.5 accompanied by any degree of hepatic encephalopathy as a result. Samples analyzed included those from 260 white patients meeting this definition, of which 79 patients were considered to have unintentional acetaminophen overdoses and 78 patients to have intentional overdoses; the remaining 103 patients had developed ALF from a variety of other causes. The definitions for these patient categories have previously been established (Schiodt et al., 1997; Larson et al., 2005; Khandelwal et al., 2011). In brief, acetaminophen toxicity patients all met clinical criteria, including alanine aminotransferase levels of ≥1000 IU/l, the presence of any level of the parent compound, acetaminophen in serum, and a history of ≥4 g per day of acetaminophen ingested, with two of three criteria qualifying for inclusion. The definition used to determine an unintentional ingestion is that acetaminophen was taken over days, with a specific cause of pain elicited and denial of suicidal intent (Schiodt et al., 1997). By contrast, patients who were considered to have taken a suicidal (intentional) overdose had taken acetaminophen at one time point, denied a cause for pain, and admitted to intent (Schiodt et al., 1997). Acute liver failure was defined as >1000 IU/l. Significant alcohol use (defined as 14 or more drinks per week) was positive for 11 donors. Complete details of donor demographics, including available medication history for individual livers, have been reported elsewhere (Court, 2010).
Genotyping. The UGT1A-3 UTR SNPs (rs10929303, c.1813C>T; rs1042640, c.1941C>G; rs8330, c.2042G>C) were genotyped by resequencing DNA obtained from the human liver bank samples (n = 48) and patients with ALF (n = 260). Briefly, PCR amplification was performed with forward primer Pri-639 (5'-GCA TAA ATT AAT CAG CCC CAC AGT GC-3') and reverse primer Pri-640 (5'-CAC CAC CCA ATT TCA TAG CAT C-3') using Platinum taq Hifi supermix (Invitrogen, Carlsbad, CA), and the resultant product was sequenced with Pri-567 (5'-GGA GCT GGA GTG ACC CTG AAT GTT C-3') (Girard et al., 2005), UGT1A6*2 allele (rs6759892, STA; rs2070959, T181A; and rs1105879, R184S) (Krishnaswamy et al., 2005b), UGT1A9 -275T (rs6759892) and UGT1A9*22 (rs45625337, T9>T) (Girard et al., 2004), and UGT2B15*2 (rs1902023, D85Y) (Court et al., 2004). Linkage disequilibrium and haplotype block analysis was performed using these SNP data and the Haplovie program (Barrett et al., 2005).

**UGT1A-3 UTR Luciferase Reporter Assays.** Plasmid luciferase 3' UTR reporter constructs containing the entire UGT1A-3 UTR reference and major variant haplotypes (CCC and TGG for rs10929303, rs1042640, and rs8330, respectively) were created using the methods previously described (Oleson et al., 2010). Human embryonic kidney 293T (HEK293T) and human colon adenoma LS180 cells were from American Type Culture Collection (Manassas, VA). Hepatocarcinoma HuH7 cells were a gift from Dr. Curt Omiecinski, Pennsylvania State University. Primary human hepatocytes in collagen coated 96-well clear-bottom, white-sided plates (Corning, Tewksbury, MA) were provided by the Liver Tissue Cell Distribution System (Stephen Strom, Department of Pathology, University of Pittsburgh, Pittsburgh, PA). Cells were from three different donors, including a 28-year-old woman, a 53-year-old woman, and a 65-year-old man. HEK293T cells were maintained in Dulbecco modified Eagle medium with high glucose and L-glutamine (Gibco/Invitrogen). The LS180 cells were from American Type Culture Collection (Manassas, VA). Cell lines and plasmids were used in the following cell types as described previously (Ge et al., 2005).

The entire UGT1A exon 3 to 5a gene region was also resequenced in selected liver samples using an additional 10 sets of primers given in Table 1. Genotypes (including methods) for the other polymorphisms in the liver bank samples were reported previously, including UGT1A1*28 (rs34815109, -53TA6) (Girard et al., 2005), UGT1A6*2 allele (rs6759892, STA; rs2070959, T181A; and rs1105879, R184S) (Krishnaswamy et al., 2005b), UGT1A9 -275T (rs6759892) and UGT1A9*22 (rs45625337, T9>T) (Girard et al., 2004), and UGT2B15*2 (rs1902023, D85Y) (Court et al., 2004). Linkage disequilibrium and haplotype block analysis was performed using these SNP data and the Haploview program (Barrett et al., 2005).

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* Segment amplified as shown in Fig. 6 numbered 5' from the 3' UTR region.
A β-gal–expressing plasmid (20 ng) was also included to control for transfection efficiency differences and nonspecific effects of plasmid overexpression as described previously (Oleson et al., 2010; Volak and Court, 2010). Three milliliters of growth media was added after 4–6 hours. Cells were harvested 48 hours after transfection, washed with phosphate-buffered saline, disrupted by gentle sonication, and the lysates assayed for acetylaspartate glucuronidation activity using 20 mM acetylaspartate, 5 mM UDP-glucuronic acid, and 3 hours of incubation time. Data were normalized to β-gal activity measured using the same lysates. Microsomes for HER293 stable cell lines expressing either UGT1A1_i1 alone or coexpressing UGT1A1_i1 and UGT1A1_i2 were obtained as previously described (Bellemare et al., 2010a). By immunoblot, the ratio of UGT1A1_i1 to UGT1A1_i2 was 1:0.33. Acetylaspartate glucuronidation activities were measured by high-performance liquid chromatography as described already herein, and the final data were normalized to the UGT1A1_i1 content of each preparation as determined by immunoblotting.

Splicing Regulatory Site Consensus Sequence Analysis. The effect of each of the UGT1A 3' UTR SNPs on binding site consensus sequences for the Srp family of splicing regulatory proteins was evaluated using the ESEfinder program (release 3.0; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (Cartegni et al., 2003). The default values of 1.956, 2.383, 2.670, and 2.676 were used as the threshold scores for the SF2/ASF, SC35, SRp40, and SRp55 consensus binding sites, respectively.

Statistical Analyses. Unless otherwise stated, statistical analyses were performed using Sigmmaplot 11 software (Systat, San Jose, CA). Associations between genotype and liver bank acetylaspartate glucuronidation activities, and between genotype and exon 5a/5b splicing ratios, were evaluated by analysis of variance (ANOVA) on log-transformed data with post-hoc pairwise testing by the Student–Newman–Keuls multiple comparisons test. Differences in allele-specific transformed data with post-hoc pairwise testing by the Student–ratios, were evaluated by analysis of variance (ANOVA) on log-concentration activities, and between genotype and exon 5a/5b splicing ratio were evaluated by Fisher's exact test. Finally, differences in UGT1A-3'UTR genotype frequencies (reference and variant carrier genotypes) between acute liver failure patients grouped by cause were evaluated by χ² test with one degree of freedom (http://hgb.gsf.de/cgi-bin/hw/hwa1.pl). A P value of less than 0.05 was considered significant.

Results

UGT1A-3'UTR SNPs Are Associated with Higher Acetylaspartate Glucuronidation Activities. SNP genotyping was conducted using DNA from our human liver bank samples focusing on genes encoding important human liver acetylaspartate glucuronidation enzymes, including UGT1A1, 1A6, and 1A9 (Bock et al., 1993; Court et al., 2001). UGT2B15 variants were also evaluated since one study showed significant acetylaspartate glucuronidation by recombinant enzyme (Mutlib et al., 2006). Genotypes were then correlated with glucuronidation activities measured in the same liver bank samples at acetylaspartate concentrations representing plasma concentrations typically associated with therapeutic use (100 μM), overdose (40 mM), and an intermediate concentration (2 mM) approximating the Kₘ value of UGT1A6, the high-affinity acetylaspartate UGT (Court et al., 2001). As shown in Table 2, only the UGT1A gene 3'-UTR SNPs (rs10929303, rs1042640, and rs8330) were associated with acetylaspartate glucuronidation activities. Interestingly, there appeared to be an acetylaspartate concentration effect on the SNP association in that rs8330 was the only SNP

<table>
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<th>Genotype</th>
<th>n</th>
<th>Mean S.D.</th>
<th>Mean S.D.</th>
<th>P Value</th>
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*P < 0.05 for ANOVA on log transformed data.

TABLE 2
Association of UGT polymorphisms with microsomal acetylaspartate glucuronidation activity measured at three different substrate concentrations in 42 human liver bank samples.
enhanced by the use of UGT1A6/UGT1A1 or UGT1A-3 genotype associations with glucuronidation activity was not the UGT1A-3 and UGT1A1 exons 1 SNPs (block 1) and the presence of two distinct haplotype blocks incorporating homozygotes.

Genetic linkage analysis of the evaluated SNPs revealed the presence of two distinct haplotype blocks incorporating the UGT1A6 and UGT1A1 SNPs (block 1) and the UGT1A-3 UTR SNPs (block 2) (Fig. 2). However, analysis of genotype associations with glucuronidation activity was not enhanced by the use of UGT1A6/UGT1A1 or UGT1A-3 UTR haplotypes compared with analysis by individual SNPs (see Supplemental Table 1).

**UGT1A-3 UTR SNPs Do Not Affect Luciferase-3’UTR Reporter Activity.** SNPs located in the 3’UTR region could affect mRNA degradation rate or translational efficiency such as through altering (creating or eliminating) response elements for microRNAs. Consequently, luciferase-3’UTR reporter constructs were generated containing each of the common UGT1A-3 UTR haplotypes (CCC-UGT1A-reference; TGG-UGT1A-variant). These were transfected into cell lines derived from three different human tissues (kidney, liver, and intestines) and also primary human hepatocytes. As shown in Fig. 3, the variant UGT1A-3 UTR did not differ in reporter activity compared with the reference UGT1A-3 UTR in all cell types examined. Interestingly, compared with constructs that lacked any of the UGT1A-3 UTRs, constructs containing the UGT1A-3 UTR (regardless of haplotype) showed 60–70% lower reporter activity in LS-180 intestinal cells (P < 0.003), whereas reporter activity was 2- to 3-fold higher with the UGT1A-3 UTR in primary human hepatocytes (P < 0.003).

**UGT1A-3 UTR SNPs May Cause Allelic Expression Imbalance.** Since the UGT1A-3 UTR SNPs are present in the mature mRNA transcript, allele-specific assay of mRNA levels can be used to determine whether there is allelic expression imbalance that would verify the presence of a cis-acting element (either the SNPs themselves or another linked variant) that would explain increased UGT1A-mediated glucuronidation. Variant to reference allele mRNA ratios were determined for 12 of the liver samples that were heterozygous for each of the three UGT1A-3 UTR SNPs (i.e., the CCC/TGG diplotype). The results showed a trend for higher variant allele mRNA levels compared with the reference allele with mean (S.D.) allele-specific ratios of 1.09 (0.18), 1.13 (0.14), and 1.14 (0.23) for rs10929303, rs1042640, and rs8330, respectively (individual data are shown in Supplemental Fig. 1). Higher variant mRNA levels were statistically significant only for rs1042640 (P = 0.007, paired t test), although rs8330 approached statistical significance (P = 0.075).

**UGT1A-3 UTR SNPs Are Associated with Increased Exon 5a to Exon 5b mRNA Variant Ratios.** Splicing of the primary UGT1A transcript can result in mRNA variants that incorporate an alternate exon 5 (termed exon 5b) that

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**UGT1A3 UTR rs8330 genotype**

![Fig. 1. Association of UGT1A-3 UTR rs8330 SNP genotype (CC, CG, and GG) with microsomal acetaminophen glucuronidation activities (40 mM acetaminophen concentration) measured in a bank of human livers obtained from 48 white donors. Shown are activities for each liver (filled circle), geometric mean values for each genotype group (horizontal dashed line), and also P values for post-hoc pairwise testing on log transformed data by the Student-Newman-Keuls multiple comparisons test (P = 0.002, ANOVA). *P < 0.05; **P < 0.01.]

**Fig. 2.** Results of pairwise linkage disequilibrium and haplotype block analysis of 9 UGT1A gene SNPs and one UGT2B15 gene SNP genotyped using DNA extracted from human liver bank samples obtained from 48 white donors. Shown above are the approximate locations of each of the nine SNPs (identified by their dbSNP rs number) relative to the unique exons 1 and the shared exons 2 to 5 in the UGT1A gene. Shown below is a matrix of linkage disequilibrium r-squared values (as percent) for each pairwise comparison. Two distinct haplotype blocks were identified, including one block incorporating the UGT1A6 and UGT1A1 SNPs (block 1) and another block incorporating the UGT1A-3 UTR SNPs (block 2).
encodes for a truncated protein (termed isoform 2 variant) that lacks the normal C-terminal trans-membrane domain (see Fig. 6B). Although these proteins are enzymatically inactive, they appear to act as repressors of enzyme activity, and it is possible that the UGT1A-3' UTR SNPs that are located in exon 5a might influence this splicing. Consequently, we measured the amount of UGT1A transcripts containing the normally spliced exon 4–5a relative to the amount of exon 4–5b alternatively spliced transcripts and determined whether this ratio was correlated with the UGT1A-3' UTR SNPs. Exon 5a/5b mRNA ratios varied greatly, ranging from as low as 0.020 (about 50-fold more exon 5b versus exon 5a) to as high as 28.2 (about 28-fold more exon 5b than exon 5a). Importantly, there was a clear association between rs8330 and exon 5a/5b mRNA ratio \((P = 0.008, \text{ANOVA})\) with about 7-fold higher mean 5a/5b ratios in rs8330 cg heterozygotes \((P = 0.013, \text{Student-Newman-Keuls test})\) and 11-fold higher in rs8330 gg homozygotes \((P = 0.017)\) compared with rs8330 cc homozygotes. Similar associations were also observed for the rs10929303 and rs1042640 SNPs (Table 3).

**UGT1A Isoform 2 Variants Are Repressors of Acetaminophen Glucuronidation.** Although UGT1A isoform 2 (UGT1A_i2) variants expressed in cell lines have been shown to decrease the glucuronidation activity of the corresponding isoform 1 (UGT1A_i1) enzymes for a number of different model substrates, effects on acetaminophen glucuronidation have not been reported. Consequently, we proceeded to evaluate the effects of UGT1A6_i2 (0–2 μg plasmid) on acetaminophen glucuronidation by UGT1A6_i1 (2 μg plasmid) transiently coexpressed in HEK293 cells. As shown in Fig. 4A, there was a profound dose-dependent effect of UGT1A6_i2 on acetaminophen glucuronidation, with nearly 90% inhibition observed with 1.5 μg or greater amounts of cotransfected plasmid \((P < 0.05, \text{repeated measures ANOVA with Holm-Sidak post-hoc testing versus control})\). Similar experiments were conducted to determine whether the UGT1A1_i2 variant also inhibited UGT1A1_i1-mediated acetaminophen glucuronidation using HEK293 cell lines that stably expressed either UGT1A1_i1 alone or both UGT1A1_i1 and UGT1A1_i2 (immunoblot ratio of 3:1). Stable expression tends to result in lower expressed protein levels that are likely closer to levels observed in human hepatocytes. UGT1A1_i2 also resulted in significant inhibition of UGT1A1_i1-mediated glucuronidation (Fig. 4B).

**TABLE 3**

<table>
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<tr>
<th>UGT1A Genotype</th>
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<th>(P) Value*</th>
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<td>11.0</td>
<td>10.8</td>
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</table>

* \(P < 0.05\) for analysis of variance on log-transformed data.
although the effect was somewhat smaller, approaching a 30% decrease ($P = 0.02, t$ test).

**UGT1A Exon 5a/5b Splicing Is Not Associated with Other Variants Between Exon 3 and Exon 5a.** The apparent effect of the 3' UTR SNPs on exon 5a versus exon 5b splicing could be explained by another genetic variant in high-linkage disequilibrium and located in an exon 5a or 5b splice regulatory site on the primary transcript. Consequently, the UGT1A gene region from exons 3–5a (including introns and exon 5b) was scanned by resequencing liver samples with high ($n = 8$) and low ($n = 8$) hepatic UGT1A exon 5a/5b mRNA splicing ratios (see Fig. 5). Seven additional variants were identified by analyzing sequence chromatograms from these samples with allele frequencies ranging from 0.13 to 0.34 (Fig. 5). All were SNPs that had been submitted previously to the dbSNP database. One additional SNP (rs1247441) referenced in dbSNP as being located in the intron between exon 5b and exon 5a showed no genetic diversity in the 16 samples assayed. Although, as expected, a clear association between each of the 3' UTR SNPs and exon 5a/5b mRNA splicing ratios ($P$, 0.006, Fisher's exact test), was found, none of the newly identified SNPs was associated with splicing phenotype ($P > 0.05$) or was in linkage with the 3' UTR SNPs.

**UGT1A-3'UTR SNPs Create Novel Splice-Enhancer Sites.** Genetic variants can modulate gene splicing through creation or abolition of splice enhancer sites. Consequently, possible effects of the UGT1A 3' UTR SNPs on binding site consensus sequences for the Srp family of splicing regulatory proteins (SF2/ASF, SC35, SRp40, and SRp55) were explored using an in silico approach. As shown in Fig. 6A, this analysis indicated that compared with the reference allele, the rs1042640 G allele variant creates a novel SRp55 site, whereas the rs8330 C allele variant creates two novel sites (SF2/ASF and SRp40). These findings suggest a model, shown in Fig. 6B, whereby the presence of the 3' UTR SNP variants favors utilization of exon 4–5a splicing over the exon 4–5b splicing pathway. This results in increased levels of the active UGT1A_i1 isoforms relative to the repressor UGT1A_i2 repressors and overall increased UGT1A-mediated glucuronidation.

![Fig. 4](https://example.com/f4.png)

**Fig. 4.** Repressive effect of UGT1A_i2 variant on UGT1A_i1-mediated acetaminophen glucuronidation activity. Top panel (A) shows acetaminophen glucuronidation activities measured in HEK293 cells transiently transfected with plasmids encoding UGT1A6_i1 (2 μg) and increasing amounts of UGT1A6_i2 (0 to 2 μg). Data were expressed as a percentage of activities in control wells that lacked UGT1A6_i2. Bars represent the mean and S.D. of three independent experiments conducted in duplicate. Also shown are the $P$ values for significant ($P < 0.05$) pairwise multiple comparisons to the control group using the Holm-Sidak test ($P = 0.02$, ANOVA). Bottom panel (B) shows acetaminophen glucuronidation activities measured in HEK293 cell lines stably expressing either UGT1A1_i1 alone or both UGT1A1_i1 and UGT1A1_i2 (ratio of 1:0.33 by immunoblot). Data were expressed as a percentage of activities in cell lines that lacked UGT1A1_i2. Bars represent the mean and S.D. of four independent experiments. Also shown is the $P$ value for the comparison in activities between cell lines by $t$ test.

![Fig. 5](https://example.com/f5.png)

**Fig. 5.** SNPs identified by resequencing DNA from human livers with high ($n = 8$) and low ($n = 8$) exon 5a to exon 5b variant mRNA ratios. Shown below are a graphic depiction of the UGT1A1 shared exons 2 to 5 region (5' to 3' with introns), the locations of primer pairs (given in Table 1) used for resequencing, and the locations of SNPs (and their dbSNP accession numbers) that were identified. Shown above are the $P$ values for a Fisher's exact test comparing SNP genotype frequencies in the low mRNA ratio versus the high mRNA ratio livers. Only the UGT1A-3' UTR SNPs (rs10929303, rs1042640, and rs8330) were significantly associated with mRNA ratios ($P < 0.05$).
UGT1A-3'UTR SNPs Are Associated with Acetaminophen-Induced Acute Liver Failure. Glucuronidation is the principal clearance mechanism for acetaminophen, and so higher acetaminophen glucuronidation would be expected to protect individuals from the well-known hepatotoxic effects of acetaminophen. Consequently, we used DNA samples from an ongoing large multicenter trial of ALF to compare UGT1A-3'UTR SNP genotype frequencies in patients who had developed ALF either unintentionally with chronic use of acetaminophen, intentionally with an acute acetaminophen overdose, or from causes other than acetaminophen. As shown in Table 4, genotype frequency differences were found between these subgroups of ALF patients. Specifically, all the UGT1A-3'UTR variant carrier genotypes were substantially underrepresented by about 2-fold in the unintentional acetaminophen hepatotoxicity subgroup compared with the other two subgroups with odds ratios (95% confidence intervals) of 0.52 (0.29–0.93, P = 0.025), 0.56 (0.31–0.99, P = 0.045), and 0.53 (0.30–0.94, P = 0.027) for rs10929303, rs1042640, and rs8330, respectively.

Rs8330 Allele Frequency Varies between Populations with Different Geographic Origin. Since the phenotypes (i.e., acetaminophen glucuronidation and risk of acetaminophen-induced hepatotoxicity) that we have associated with the rs8330 SNP could vary between populations in relation to their geographic origin, we compared the minor allele frequencies determined for rs8330 in this study to available published and database frequencies reported for other populations. As shown in Table 5, substantial geographic differences with low frequencies were observed for East Asian populations (0.11–0.16), with high frequencies for African populations (0.39 and 0.50) and intermediate frequencies for ancestral European (“white”) populations (0.21–0.25). Consistent with the reported race or ethnicity of the DNA sample donors used in this study, allele frequencies for the human liver bank samples (0.21), the intentional acetaminophen-induced ALF subjects (0.22), and the subjects with ALF from other causes (0.26) most closely resembled those of other European ancestral populations, whereas the unintentional acetaminophen-induced ALF subjects had a considerably lower frequency (0.16).

Discussion
To our knowledge, this is the first study to identify genetic polymorphisms associated with interindividual variability in acetaminophen glucuronidation in the human

<table>
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<th>Genotype</th>
<th>Unintentional</th>
<th>Intentional</th>
<th>Other Causes</th>
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<tr>
<td>rs10929303</td>
<td>C/C</td>
<td>57 (72)</td>
<td>*0.025</td>
</tr>
<tr>
<td>rs10929303</td>
<td>C/T+T/T</td>
<td>22 (28)</td>
<td>36 (46)</td>
</tr>
<tr>
<td>rs8330</td>
<td>C/C</td>
<td>56 (71)</td>
<td>*0.027</td>
</tr>
<tr>
<td>rs8330</td>
<td>C/G+G/G</td>
<td>23 (29)</td>
<td>36 (46)</td>
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</table>
liver. Of the SNPs evaluated, only those located in the UGT1A-3’UTR were associated with variable acetaminophen glucuronidation. The UGT1A-3’UTR SNPs, rather than those located within individual UGT1A isoform genes, have the greatest potential to impact the disposition of drugs (such as acetaminophen) that are glucuronidated by multiple UGT1A isoforms, since SNPs affecting only an individual UGT1A enzyme could be compensated for by acetaminophen glucuronidation by other unaffected UGT1A isoforms. Of the three UGT1A-3’UTR SNPs, rs8330 was most consistently associated with glucuronidation phenotype at all three acetaminophen concentrations tested (0.1, 2, and 40 mM). Consequently, this SNP is likely to be the most robust biomarker of acetaminophen glucuronidation over a wide range of acetaminophen dosages (clinical to toxic).

Few studies have evaluated the association between the UGT1A-3’UTR SNPs and UGT1A glucuronidation phenotype. A prior study comprising 85 Japanese cancer patients receiving irinotecan found a trend for higher total bilirubin levels in individuals with the variant 3’UTR TGG haplotype (termed *1B) after stratifying by UGT1A1*28 genotype, presumably reflecting decreased UGT1A1-mediated bilirubin glucuronidation (Saiki et al., 2006). However, in the same subjects, UGT1A haplotype had no effect on metabolic ratios of SN-38 glucuronide to SN-38, an irinotecan metabolite glucuronidated by hepatic UGT1A1. Furthermore, in a somewhat larger study of 125 African American and white women, rs10929303 (c.1813C>T) was not associated with altered bilirubin levels, even after controlling for UGT1A1*28 genotype effect (Hong et al., 2007). Finally, a weak (although statistically significant) association was reported between a UGT1A-3’UTR SNP and elevated liver function tests in patients taking tolcapone, presumably because of decreased tolcapone glucuronidation (Acuna et al., 2002). Unfortunately, the exact identity of this SNP is unclear since it was given as “C908G” in the report and could be either rs1042640 or rs8330.

None of the UGT1A isoform-specific SNPs evaluated here was associated with altered hepatic acetaminophen glucuronidation. This finding is in agreement with the results of a pharmacokinetic study that showed no association of UGT1A1*28 genotype with urinary acetaminophen glucuronide to acetaminophen ratios in 23 healthy male subjects (Rauchschwalbe et al., 2004). However, another study in patients with β-thalassemia showed decreased acetaminophen area under the plasma concentration-time curve, presumably reflecting increased clearance by glucuronidation, in patients carrying UGT1A6*2 and lacking UGT1A1*28 compared with subjects who carried neither variant allele (Tankanitlert et al., 2007). Unfortunately, that study was limited by small sample size (only five subjects per group), possible effect of disease, and also conflicting evidence in that both plasma acetaminophen glucuronide and sulfate plasma concentration-time curves were also decreased in the UGT1A6*2 carriers, which is not consistent with enhanced glucuronidation. Finally, a recent study in 66 healthy subjects showed a trend for increased urinary acetaminophen glucuronide to parent ratios (by almost 30%) in UGT1A6*2 carriers, which is not consistent with enhanced glucuronidation. However, another study in patients carrying UGT1A6*2 and lacking UGT1A1*28 showed no association of this allele with urinary acetaminophen glucuronidation. This finding is in agreement with the results of the present study, where these weak associations could be the result of partial linkage disequilibrium.

### Table 5

Comparison of rs8330 minor allele frequencies (MAF) determined for subjects in the current study with published or data base values for different human populations

<table>
<thead>
<tr>
<th>Sample Source</th>
<th>Population</th>
<th>No. of Alleles</th>
<th>MAF</th>
<th>Reference</th>
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<td>0.16</td>
<td>This study</td>
</tr>
<tr>
<td>Intentional acetaminophen -induced acute liver failure patients</td>
<td>White American</td>
<td>158</td>
<td>0.26</td>
<td>This study</td>
</tr>
<tr>
<td>Acute liver failure patients from Tufts liver bank samples</td>
<td>White American</td>
<td>206</td>
<td>0.22</td>
<td>This study</td>
</tr>
<tr>
<td>French Canadian cohort</td>
<td>European</td>
<td>508</td>
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<td>Menard et al., 2009</td>
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</table>

**SNP:** single nucleotide polymorphism.


**1000 Genomes Project data:** http://browser.1000genomes.org.
reported in that study, and so it was not possible to calculate partial metabolite clearances, which may be a more independent index of metabolic capacity.

The association of acetaminophen glucuronidation with genotype was strongest for the rs8330, suggesting that rs8330 might be the causal SNP rather than another linked variant on the same UGT1A-3’UTR haplotype block. Consequently, we conducted a series of mechanistic studies to explore this hypothesis. We found no evidence that the UGT1A-3’UTR SNPs influence mRNA stability or translation efficiency such as through altering binding sites for microRNAs or other post-transcriptional regulators. However, allele-specific assay of heterozygous liver RNA suggested that there was a statistically significant (albeit small) imbalance with higher variant versus reference allele mRNA levels. This observation was further corroborated by quantitation of UGT1A exon 5 mRNA, which indicated that the rs8330-containing allele was associated with increased levels of exon 5a versus exon 5b mRNA transcripts.

Exon 5b containing UGT1A transcripts encode for enzymatically inactive truncated proteins lacking the C-terminal transmembrane domain (termed isoform 2 variant or i2), which might play a role in the regulation of the isoform 1 (i1) enzyme through hetero-oligomerization and repression (see proposed model in Fig. 6B). This contention is supported by our i1/i2 coexpression studies that showed a clear repressive effect of coexpression of the i2 variant on acetaminophen glucuronidation mediated by both of the i1 forms of UGT1A1 and UGT1A6. A prior study also showed about 60% lower deferiprone glucuronidation in HEK293 cells stably expressing UGT1A6i1 plus UGT1A6i2 versus UGT1A6i1 alone (normalized by i1 immunoblot content) (Benoit-Biancamano et al., 2009). Although a recent paper (Jones et al., 2012) suggests that UGT1A i2 levels may be too low in human liver to affect UGT1A i1 activity, these authors measured only mRNA levels, and it is unclear whether i2 protein levels are correspondingly low. Interestingly, they did show significant enhancement of raloxifene glucuronidation in HepG2 (hepatocellular carcinoma G2) cells by siRNA-mediated knockdown of UGT1A i2 mRNA. An earlier study also using siRNA-mediated knockdown of endogenous i2 also demonstrated upregulation of cellular glucuronidation activities in colon cancer cells for various substrates of UGT1A enzymes (Bellemare et al., 2010b).

With regard to the mechanism by which the UGT1A-3’UTR SNPs might regulate exon 5a/5b splicing, our in silico analysis indicated that these SNPs alter predicted consensus binding elements for splice regulatory enhancer proteins, with the greatest effect observed for rs8330. Further gene sequencing failed to identify another gene variant that could affect exon 5 splicing, although it is possible that such a variant may exist either upstream of exon 3 or downstream of exon 5a. Although beyond the scope of the current work, a direct method to prove the role of one or more of the 3’UTR SNPs in regulating UGT1A splicing would be through analysis of allelic variants of exon-containing minigene constructs, as was recently reported for the CYP2B6 gene (Hofmann et al., 2008).

This is also the first study to identify genetic variants associated with susceptibility to ALF in patients with a history of chronically consuming large amounts of acetaminophen. The lower frequency of the UGT1A-3’UTR genotypes in these patients is consistent with a protective effect of the variant allele resulting from more extensive detoxification of acetaminophen via glucuronidation. However, we did not observe this association in patients who had developed ALF from intentional acetaminophen overdose. One possible reason for this difference is that the intentional acetaminophen overdose patients tended to ingest quite large amounts of acetaminophen (median of 26 g per patient in this study) as a single dose such that detoxification via glucuronidation may have been overwhelmed regardless of genotype. In contrast, although the unintentional toxicity patients tended to consume even higher total amounts of acetaminophen (median of 45 g per patient in this study), they did so over a longer period (median of 7 days in this study), resulting in a lower daily dose (median of 6.5 g per day) that might not have overwhelmed the glucuronidation pathway, allowing for an influence of the rs8330 variant.

A weakness of the current study is that we were unable to identify an appropriate comparator group, such as individuals who had consumed similar doses of acetaminophen over a similar time course as the unintentional acetaminophen toxicity patients had but had not subsequently developed ALF. However, comparisons with published data suggested that the rs8330 allele frequency in the unintentional acetaminophen toxicity patients was lower than expected for a white population of European origin. Compared with European populations, data for rs8330 also predict that African populations should have higher acetaminophen glucuronidation rates associated with a higher rs8330 frequency, whereas East Asian populations with a lower rs8330 frequency should have lower acetaminophen glucuronidation rates. Interestingly, higher acetaminophen glucuronidation rates have been reported for Ghanaian (West African) and Kenyan (East African) versus white populations (Critchley et al., 1986); no differences were reported in another study of a Venda (South African) versus a white population (Sommers et al., 1987). Lower acetaminophen glucuronidation was reported in Hong Kong Chinese versus white subjects in one study (Critchley et al., 2005), although differences (versus whites) were not observed in a study of Chinese living in Australia (Osborne et al., 1991) or for “Orientals” living in Canada (Patel et al., 1992). Further studies are needed to clarify the relationship between genetic polymorphisms, acetaminophen metabolism, and susceptibility to acetaminophen toxicity in different human populations.
Supplemental Figure 1

Journal name: Journal of Pharmacology and Experimental Therapeutics

Article title: The UDP-glucuronosyltransferase (UGT) 1A polymorphism c.2042C>G (rs8330) is associated with increased human liver acetaminophen glucuronidation, increased UGT1A exon 5a/5b splice variant mRNA ratio, and decreased risk of unintentional acetaminophen-induced acute liver failure.

Authors: Michael H. Court, Marina Freytsis, Xueding Wang, Inga Peter, Chantal Guillemette, Suwagmanzi Hazarika, Su X. Duan, David J. Greenblatt, William M. Lee and the Acute Liver Failure Study Group.

Suppl. Fig. 1. Allelic expression imbalance ratios in 12 human liver samples that were heterozygous for the UGT1A-3'UTR SNPs rs10929303, rs1042640, and rs8330 (reference alleles given in the Methods section). Shown are the ratios of variant to reference allele mRNA levels, normalized to gDNA levels for each of the 12 human liver samples. The dotted line indicates an allelic expression ratio of 1.0 that would be expected if there was no difference in normalized mRNA levels between the alleles.
Supplemental Table 1. Association of UGT haplotypes with microsomal acetaminophen glucuronidation activity measured at 3 different substrate concentrations in human liver bank samples. Haplotypes evaluated included the UGT1A-3'UTR haplotype with SNPs c.1813C>T, c.1941C>G, and c.2042C>G; and the UGT1A6/UGT1A1 haplotype with variants UGT1A6 c.19T>G, c.541A>G, c.552A>C, and UGT1A1 -53TA6>TA7.

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<td>2839</td>
<td>1292</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05 for ANOVA on log transformed data.