Candidate Gene Polymorphisms in Patients with Acetaminophen-Induced Acute Liver Failure.

Michael H. Court, Inga Peter, Suwagmani Hazarika, Magdalini Vasiadi, David J. Greenblatt, William M. Lee and the Acute Liver Failure Study Group.

Pharmacogenomics Laboratory (M.H.C.), Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, Washington, USA; Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA (I.P.); Department of Molecular Physiology and Pharmacology (S.H. and D.J.G.), Tufts University School of Medicine, Boston, Massachusetts, USA; Program in Pharmacology and Experimental Therapeutics (M.V., D.J.G.), Tufts University Sackler School of Graduate Biomedical Sciences, Boston, Massachusetts, USA; and the Department of Internal Medicine (W.M.L.), University of Texas Southwestern Medical Center, Dallas, Texas, USA.

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Corresponding author: Michael H. Court, BVSc, PhD, Pharmacogenomics Laboratory, Department of Veterinary Clinical Sciences, Washington State University College of Veterinary Medicine, 100 Grimes Way, Pullman, WA 99163, USA. Telephone: 509-335-0817; Fax: 509-335-0880; Email: michael.court@vetmed.wsu.edu.

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ABSTRACT

Acetaminophen is a leading cause of acute liver failure (ALF). Genetic differences might predispose some individuals to develop ALF. In this exploratory study we evaluated genotype frequency differences among patients enrolled by the ALF Study Group that had developed ALF either intentionally from a single time point overdose of acetaminophen (N=78), unintentionally following chronic high doses of acetaminophen (N=79), or from causes other than acetaminophen (N=103). Polymorphisms evaluated included those in genes encoding putative acetaminophen metabolizing enzymes (*UGT1A1, UGT1A6, UGT1A9, UGT2B15, SULT1A1, CYP2E1* and *CYP3A5*) as well as *CD44* and *BHMT1*. Individuals carrying the *CYP3A5* rs776746 A allele were overrepresented among ALF patients that had intentionally overdosed with acetaminophen with an odds ratio of 2.3 (95% confidence interval: 1.1 – 4.9, *P*=0.034) compared with all other ALF patients. This finding is consistent with enhanced bioactivation of acetaminophen by the CYP3A5 enzyme. Persons homozygous for the *CD44* rs1467558 A allele were also overrepresented among patients that had unintentionally developed ALF from chronic acetaminophen use with an odds ratio of 4.0 (1.0 – 17.2, *P*=0.045) compared with all other ALF subjects. This finding confirms a prior study that found elevated serum liver enzyme levels in healthy volunteers with the CD44 rs1467558 AA genotype that had consumed high doses of acetaminophen for up to 2 weeks. However both genetic associations were considered relatively weak and were not statistically significant after adjustment for multiple comparisons testing. Nevertheless, both CYP3A5 rs776746 and CD44 rs1467558 warrant further investigation as potential genomic markers of enhanced risk of acetaminophen-induced ALF.

INTRODUCTION

Acetaminophen is one of the most widely available and commonly used pain reliever and fever reducing drugs. Unfortunately this drug is also one of the leading causes of acute liver failure in this country, accounting for approximately half of all acute liver failure cases identified by the Acute Liver Failure Study Group in the United States (Larson et al., 2005). In most cases liver injury results from an intentional single time point overdose of acetaminophen, but in a substantial number of cases, injury was unintentional and acetaminophen was consumed over a more prolonged time period.

Genetic variability could predispose some individuals to a higher risk of acetaminophen-induced liver injury than would be predicted based on the dose ingested, leading to acute liver failure in a subset of these patients (Patel et al., 1992; Court et al., 2001; Rauchschwalbe et al., 2004; Zhao and Pickering, 2011). Candidate genes for this genetic variability would include those encoding enzymes important in the clearance of acetaminophen, including the UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), and also the cytochrome P450 enzymes (CYPs) responsible for converting acetaminophen to the hepatotoxic N-acetylparaquinoneimine (NAPQI). In a recent study we identified a polymorphism in the UDP-glucuronosyltransferase (UGT) 1A gene (rs8330) associated with an increased rate of acetaminophen detoxification by hepatic glucuronidation and a decreased risk of unintentional acetaminophen-induced liver failure (Court et al., 2013). This polymorphism is likely to have a broad impact on drug glucuronidation since it is located in the 3'untranslated region of the UGT1A gene that is shared through differential gene splicing by all 9 transcribed UGT1A isoforms, including all hepatic isoforms that have been shown to glucuronidate acetaminophen (UGT1A1, 1A6 and 1A9).

Here we have used DNA samples from patients enrolled by the Acute Liver Failure Study Group to evaluate associations of other polymorphisms in genes encoding the acetaminophen detoxification enzymes UGTs 1A1, 1A6, 1A9, 2B15, and SULT1A1, as well as putative NAPQI forming enzymes CYPs 2E1 and 3A5.   We also evaluated a common nonsynonymous polymorphism in the human CD44 gene (I479T) that was associated with increased serum liver injury biomarker levels in healthy volunteers who chronically consumed acetaminophen (Watkins et al., 2006; Harrill et al., 2009), and also a common non-synonymous polymorphism in the human betaine-homocysteine methyltransferase 1 (BHMT1) gene (R239Q) since the closely related murine *Bhmt2* gene was previously identified as protective against acetaminophen-induced hepatotoxicity in a mouse genomics study (Liu et al., 2010).

MATERIALS AND METHODS

Acute liver failure patient DNA samples

DNA samples were obtained with appropriate consent from patients enrolled by the Acute Liver Failure Study Group, a consortium of US liver centres established in 1998 to better define causes and outcomes of acute liver failure (ALF) (Ostapowicz et al., 2002).   Standard entry criteria for acute liver failure were employed.   All patients were deemed to have had an acute hepatic injury of less than 26 weeks duration and demonstrated an international normalized ratio (INR) of ≥ 1.5 accompanied by any degree of hepatic encephalopathy as a result. Samples analyzed included those from 260 white patients meeting the above definition, of which 79 patients were considered to have unintentional acetaminophen overdoses and 78 patients to have intentional overdoses, while 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, assigning acetaminophen as the cause of ALF required meeting clinical criteria including a history of ingesting more than 4 gm of acetaminophen per day within 7 days of admission, the presence of any level of acetaminophen in serum, and significantly elevated alanine aminotransferase (ALT) levels of $\geq$ 1000 IU/L, with 2 of 3 criteria qualifying for inclusion. Exclusion of other potential causes of ALF (listed below) was also required. 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 the acetaminophen at one time point, denied a cause for pain and admitted to intent of self-harm. Etiologies for the other 103 patients varied greatly, including indeterminate (27), drug-induced liver injury (23), shock/ischemia (15), hepatitis B (11), autoimmune hepatitis (8), other viruses (3), acute fatty liver of pregnancy (2), Budd-Chiari syndrome (2), hepatitis A (2), hepatitis E (1), Wilson's disease (1), mushroom ingestion (1), and other causes (7).

Genotyping

Methods for genotyping the UGT polymorphisms have been described previously, including UGT1A1 -53(TA) x 5,6,7 or 8 (Girard et al., 2005), UGT1A6 S7A (rs6759892), T181A (rs2070959), and R184S (rs1105879) (Krishnaswamy et al., 2005), UGT1A9 -275T>A (rs6714486) (Girard et al., 2004), UGT2B15*2 (rs1902023, D85Y) (Court et al., 2004), and UGT1A-3'UTR c.2042C>G (rs8330) (Court et al., 2013). Taqman® allele discrimination assays were used to genotype DNA samples on a ABI 7300 real-time PCR instrument for the CYP3A5*3 (rs776746; ABI assay C_26201809-30), CD44 I479T (rs1467558; ABI assay C_2143203_10), and BHMT1 R239Q (rs3733890; ABI assay C_11646606_20) SNPs. CYP2E1

copy number variation (CYP2E1*1x2) was also determined on a ABI 7300 real-time PCR instrument with the Taqman® copy number variation assay (ABI assay Hs00231786_cn) using RNase P (ABI assay 4403326) as the reference gene. Since reference allele frequency data were not available for CYP2E1*1x2, DNA samples obtained with appropriate consent from 48 United States white liver bank donors (donor details given in (Court, 2010)) were assayed in addition to the acute liver failure study subject samples. The CYP2E1*1D tandem repeat polymorphism (6 repeats in the C allele and 8 repeats in the D allele) was assayed by PCR with agarose gel sizing according to the method described previously (Hu et al., 1999) with modified PCR primers including 5'-TGG TAC ATT GTG AGA CAG TG-3' (Pri-745) and 5'-ATA CGG GAA CAC CTC GTT TG-3' (Pri-746). PCR product sizes for the C and D alleles were 610 bp and 700 bp, respectively. The SULT1A1*2 polymorphism (rs9282861, R213H) was assayed by the PCR-RFLP method described previously (Wang et al., 2002) with modified primers including 5'-TCA GTA ATC CGA GCC TCC AC-3' (Pri-741) and 5'-GTT TGA TTC GCA CAC TCC CT-3' (Pri-742). *HaeII* digestion of PCR product yielded 309 bp and 393 bp fragments for the *1 and *2 alleles, respectively. The genotypes of representative samples were confirmed by direct sequencing of PCR product.

Statistical analyses

The genotype frequencies of each acute liver failure patient group (unintentional acetaminophen, intentional acetaminophen, or other causes) were compared to the combined genotype frequencies for the other two acute liver failure groups by Chi-squared test with one degree of freedom (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl accessed 25 June 2013). Both recessive (combining homozygous reference and heterozygote genotype numbers) and dominant (combining heterozygote and homozygous variant genotype numbers) genetic models were

evaluated to enhance statistical power. Allele frequency differences between acute liver failure

patient groups were also evaluated in a similar manner by comparing the allele frequency of one

group with the combined allele frequency of the other two acute liver failure groups. A p value

of less than 0.05 was considered significant. For statistical analysis purposes, the UGT1A1

promoter repeat expansion polymorphism consisting of either 5, 6, 7, or 8 TA repeats (i.e. 4

possible alleles) was reduced to 2 effective allele groups of either 5 or 6 repeats (fast metabolizer

alleles) or 7 or 8 repeats (slow metabolizer alleles).

RESULTS AND DISCUSSION

The numbers of ALF patients with each of the assayed genotypes are given in Table 1

grouped by ALF etiology. None of the genotype frequencies evaluated were found to deviate

from the expected Hardy-Weinberg equilibrium values. Allele frequencies for these

polymorphisms are shown in Figure 1 to allow for comparisons with published or database allele

frequencies for a race/ethnicity matched population. We elected to analyse the acetaminophen-

induced ALF patients as either one group (versus the non-acetaminophen ALF patients) or as 2

separate groups based upon intent (intentional or unintentional hepatotoxicity). As we reported

previously (Court et al., 2013), although the 2 ALF groups consumed essentially the same total

dose of acetaminophen, the intentional group did so at a single time point, while the

unintentional group had consumed the acetaminophen over a period of days to weeks, such that

daily dose was lower, and there was an opportunity for adaptive changes. Consequently, a

distinct but potentially overlapping set of genes could influence risk of hepatotoxicity in these

ALF groups.

As reported in preliminary form previously (Court et al., 2013), the UGT1A-3'UTR rs8330 variant carrier genotype frequency was significantly lower in the unintentional acetaminophen hepatotoxicity group (29%) as compared with the other ALF subgroups (44%) with an odds ratio (95% confidence interval) of 0.53 (0.30-0.94, $P = 0.027$). This finding is consistent with a protective effect of the variant rs8330 G allele through enhancement of acetaminophen glucuronidation and detoxification as demonstrated by a series of *in vitro* studies (Court et al., 2013). Conversely it would imply that non-carriers of this allele (i.e. rs8330 CC genotype) would be at increased risk of unintentional acetaminophen hepatotoxicity.   The only other polymorphism associated with the unintentional acetaminophen-induced ALF group was CD44 rs1467558, with patients homozygous for the CD44 variant A allele being overrepresented with an odds ratio of 4.0 (1.0-17.2, $P = 0.045$). This finding is consistent with a prior study that showed an association of this same polymorphism with elevated serum alanine aminotransferase levels (a biomarker of hepatocellular injury) in healthy volunteers that consumed the maximum recommended dose of acetaminophen for up to 2 weeks (Watkins et al., 2006; Harrill et al., 2009). However, since acetaminophen dosing was stopped in that study once significant liver enzyme elevations were detected in the affected subjects, it is not clear whether those subjects would have proceeded to develop fulminant hepatic failure with continued drug exposure.

Given that both UGT1A-3'UTR rs8330 and CD44 rs1467558 might additively contribute to risk for acetaminophen-induced ALF, we also determined the numbers of subjects who had both of the risk genotypes (rs8330 CC and rs1467558 TT).   Interestingly, 5 of 156 acetaminophen-induced ALF patients (3 of 79 unintentional, and 2 of 78 intentional) had both of these risk alleles, while none of the 103 non-acetaminophen ALF patients had both these alleles.

Unfortunately the relative small number of patients with both genotypes precluded any definitive statistical analysis.

The only other polymorphism that appeared to influence risk for acetaminophen-induced hepatotoxicity was CYP3A5 rs776746, with the A allele being more frequently observed in the intentional acetaminophen-induced ALF patients relative to patients with ALF from other causes, with an OR of 2.3 (1.1 - 4.9, P = 0.034). This association is consistent with enhanced formation of NAPQI from acetaminophen by CYP3A5 in individuals carrying the rs776746 A allele, in contrast to individuals with the G allele who lack active CYP3A5 enzyme because of aberrant gene splicing (Kuehl et al., 2001). Although the capacity of CYP3A5 to oxidize acetaminophen has not been reported, a recent study showed that recombinant CYP3A4 had the highest acetaminophen bioactivation capacity out of 9 human hepatic CYPs tested (other than CYP3A5), including CYP2E1 and CYP1A2 (Laine et al., 2009). Given the high degree of overlap in substrate selectivity between CYP3A enzymes, it is likely that CYP3A5 could oxidize acetaminophen, and this hypothesis should be tested in future studies.

UGT1A1 -53TA(n) also appeared to differ between ALF groups in that there was a higher frequency of carriers of the slow glucuronidation UGT1A1 alleles (7 or 8 TA repeats) in the non-acetaminophen ALF patients (66 of 101 patients; 65%) as compared with patients with acetaminophen-induced ALF (75 of 157 patients; 48%) with an OR of 2.0 (1.2 – 3.4, P = 0.0066). We recently reported a UGT1A1 slow metabolizer genotype frequency of 48% (20 of 44 subjects) for white subjects within our human liver bank samples (Yasar et al., 2013), suggesting that the acetaminophen-induced ALF patients probably do not differ in the frequency of this polymorphism from a race and ethnicity matched population, while the non-acetaminophen ALF patients probably have an unexpectedly higher frequency of slow UGT1A1

glucuronidation carriers. Further stratification of the non-acetaminophen ALF cases by etiology showed the highest frequencies of the UGT1A1 poor metabolizer genotypes in patients with ALF from shock (12 of 15 patients; 80%), from indeterminate causes (18 of 26 patients; 69%), and from drug-induced hepatitis (14 of 22 patients; 64%).

Slower glucuronidation by UGT1A1 of drugs (other than acetaminophen) administered to these patients may have contributed to the pathogenesis of ALF in these patients. However, a review of the drugs implicated as a cause ALF in the slow metabolizer patients, identified only one drug known to be cleared by glucuronidation (valproate),   It is also possible that slower glucuronidation of bilirubin by UGT1A1 (the only enzyme capable of conjugating bilirubin) in carriers of the slow metabolizer UGT1A1 genotypes might also have enhanced the development of symptoms of jaundice in the non-acetaminophen ALF patients.

There are several limitations to the current study that should be pointed out. We were unable to use a classical case-control study design since we did not have access to DNA samples from a control population that had received a similar acetaminophen dose (acute or chronic) to the ALF patients, but had not developed ALF. Consequently our approach may have limited the statistical power to discern genotype frequency differences. The numbers of DNA samples studied was also relatively small for a genetic association study of this type, although we used all the available ALF patient DNA samples. Furthermore, we tested different genotypes for frequency differences between groups which would tend to increase the type I (false positive) error rate, and so a P value of 0.05 is probably not stringent enough for significance. Consequently, our results should be considered exploratory and will need to be confirmed by analysis of other larger patient populations.

In conclusion, we have identified polymorphisms in the CD44 (rs1467558) and CYP3A5 (rs776746) genes that may contribute to risk for acetaminophen-induced ALF, in addition to the previously discovered UGT1A-3'UTR variant (rs8330). These SNPs warrant further investigation as potential genomic markers of enhanced risk of acetaminophen-induced ALF.

AUTHOR CONTRIBUTIONS

Participated in research design: Court, Lee, Greenblatt, Rossaro, Munoz, Shaikh, Crippin, McGuire, Chung, Fix, McCashland, Ganger, Fontana, Brown, Schilsky, Satyanarayana, Reuben, Liou, Hynan, Sanders, Durkalski, Dillon, Battenhouse, Attar, Pezzia, Samuel.

Conducted experiments: Court, Vasiadi, Hazarika, Zaman, Rossaro, Munoz, Larson, Murray, Smith, Brown, Satyanarayana.

Contributed new reagents or analytic tools: Greenblatt, Stravitz, Crippin, Harrison, Reddy, Zhao.

Performed data analysis: Court, Vasiadi.

Wrote or contributed to the writing of the manuscript: Court, Lee, Greenblatt, Peter, Rossaro, Han, Smith, Fontana.

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FOOTNOTES

FIGURE LEGENDS

Figure 1. Allele frequencies for candidate genes potentially involved in the pathogenesis of acetaminophen-induced acute liver failure (APAP-ALF). Shown are the frequencies determined for 261 patients who had developed ALF either unintentionally from chronic acetaminophen use (N = 79), intentionally from a single time point acetaminophen overdose (N =78), or from causes other than acetaminophen (N = 103). Chi-squared analysis showed a higher allele frequency (*$P<0.05$) for the CYP3A5 fast metabolizer rs776746 G allele in patients that had a single time point acetaminophen overdose, and for UGT1A1 slow glucuronidation -53(TA)7or8 alleles in the patients with ALF from causes other than acetaminophen when compared to the other 2 ALF groups. Also shown for comparison are allele frequencies obtained from the NCBI database for a race/ethnicity matched white population (60 Utah residents with Northern and Western European ancestry), except for CYP2E1*1x2 copy number variation, which were determined in this study using DNA from 42 white individuals (see Methods section).

Table 1. Comparison of genotype frequencies in 260 patients who had developed acute liver failure either unintentionally from chronic acetaminophen use (N = 79), intentionally from acute acetaminophen overdose (N = 78), or from causes other than acetaminophen (N = 103).

Genotype		Acetaminophen						Other causes		
		Unintentional			Intentional					
		N	(%)	[a]P values	N	(%)	[a]P values	N	(%)	[a]P values
UGT1A-3'UTR	C/C	56	(71)	*0.027, 0.98	42	(54)	0.13, 0.48	60	(58)	0.50, 0.53
rs8330	C/G	20	(25)		32	(41)		40	(39)	
	G/G	3	(4)		4	(5)		3	(3)	
UGT1A1 (TA)n	56/56	39	(50)	0.28, 0.71	42	(54)	0.064, 0.33	35	(35)	*0.0066, 0.56
5or6->7or8	56/78	30	(38)		30	(38)		54	(53)	
	78/78	9	(12)		6	(8)		12	(12)	
UGT1A6	T/T	32	(41)	0.13, 0.87	28	(36)	0.58, 0.67	26	(27)	0.050, 0.58
rs6759892	T/G	35	(44)		38	(49)		55	(56)	
	G/G	12	(15)		11	(14)		17	(17)	
UGT1A6	A/A	40	(51)	0.16, 0.38	36	(47)	0.57, 0.35	36	(37)	0.061, 0.94
rs2070959	A/G	30	(38)		36	(47)		53	(54)	
	G/G	9	(11)		5	(6)		9	(9)	
UGT1A6	A/A	37	(47)	0.23, 0.39	35	(45)	0.38, 0.19	33	(34)	*0.049, 0.68
rs1105879	A/C	32	(41)		37	(48)		54	(55)	
	C/C	10	(13)		5	(6)		11	(11)	
UGT1A9	T/T	72	(91)	0.44	66	(85)	0.16	93	(90)	0.55
rs6714486	T/A	7	(9)		12	(15)		10	(10)	
UGT2B15	G/G	21	(30)	0.62, 0.45	19	(25)	0.58, 0.06	27	(28)	0.95, 0.29
rs1902023	G/T	36	(51)		34	(45)		52	(53)	
	T/T	14	(20)		23	(30)		19	(19)	
SULT1A1	G/G	36	(46)	0.87, 0.64	34	(44)	0.89, 0.94	46	(45)	0.97, 0.61
rs9282861	G/A	35	(44)		36	(47)		49	(48)	
	A/A	8	(10)		7	(9)		8	(8)	
CYP2E1*1D	6/6	75	(96)	0.83	77	(99)	0.21	98	(95)	0.32
6->8 repeats	6/8	3	(4)		1	(1)		5	(5)	
CYP2E1*1x2	1/1	74	(94)	0.94, 0.13	74	(95)	0.65, 0.51	96	(93)	0.73, 0.42
1->2 copies	1/2	4	(5)		4	(5)		7	(7)	
	2/2	1	(1)		0	(0)		0	(0)	
CYP3A5	G/G	74	(94)	0.082	64	(82)	*0.034	92	(89)	0.73
rs776746	G/A	5	(6)		14	(18)		11	(11)	
CD44	G/G	51	(65)	0.21, *0.045	59	(76)	0.19, 0.75	72	(70)	0.98, 0.11
rs1467558	G/A	23	(29)		17	(22)		30	(29)	
	A/A	5	(6)		2	(3)		1	(1)	
BHMT1	G/G	34	(43)	0.56, 0.41	31	(40)	0.20, 0.10	54	(52)	0.081, 0.43
rs3733890	G/A	37	(47)		33	(42)		38	(37)	
	A/A	8	(10)		14	(18)		11	(11)	

[a] *P* values are given for Chi-squared test of both dominant (left value) and recessive (right value) genetic models comparing differences in genotype numbers for the indicated acute liver failure group with the summed genotype numbers for the other two acute liver failure groups. Only one *P* value is listed for the UGT1A9, CYP2E1*1D, and CYP3A5 polymorphisms since no homozygous variant genotype individuals were found, and so only the dominant model could be tested. *$P<0.05$

Figure 1