Functional polymorphisms of UDP-glucuronosyltransferases *1A1, 1A6* and *1A8* are not involved in chronic pancreatitis

Mariette Verlaan[a], René H.M. te Morsche[a], Akos Pap[b], Robert J.F. Laheij[a], Jan B.M.J. Jansen[a], Wilbert H.M. Peters[a] and Joost P.H. Drenth[a]

Objectives Chronic pancreatitis (CP) is associated with alcohol abuse, smoking and other dietary or environmental factors. UDP-glucuronosyltransferases (UGTs) are phase II detoxifying enzymes responsible for glucuronidation of various exogenous and endogenous compounds. Genetic variations, resulting in variable rates of glucuronidation, are of toxicological and physiological importance and are frequently associated with diseases. Recently, a genetic polymorphism in UGT1A7 was possibly associated with an increased risk for CP. We investigated whether polymorphisms in the genes for UGT1A1, UGT1A6 and UGT1A8 modified the risk for CP.

Methods DNA samples were obtained from 258 adult CP patients with alcoholic ($n = 153$), hereditary ($n = 25$) or idiopathic ($n = 80$) origin. DNA from 140 healthy controls was analyzed for comparison. Patients and controls were all of Caucasian origin. Genetic polymorphisms in UGTs were determined by PCR, eventually followed by restriction-fragment-length-polymorphism analyses in all subjects.

Results The distribution of the various alleles of *UGT1A1*, *UGT1A6* and *UGT1A8* did not differ between CP patients and healthy controls.

Conclusion These data suggest that genetic polymorphisms in *UGT1A1*, *UGT1A6* and in *UGT1A8* do not predispose to the development of CP in Caucasians. *Pharmacogenetics* 14:351–357 © 2004 Lippincott Williams & Wilkins

Introduction

Chronic pancreatitis (CP) is a progressive inflammatory disease, which eventually leads to functional impairment of exocrine and/or endocrine function of the organ [1–4]. The causes of CP are multifaceted. Most cases have been attributed to alcohol abuse, but other etiological factors such as heredity, smoking, anatomical variations, and various metabolic disorders have also been identified [5].

The elucidation of genes involved in hereditary forms of CP has shed some insight in the pathogenesis of the disorder [6]. In autosomal dominant forms of hereditary CP, mutations in the cationic trypsinogen gene are linked to the disease, whereas in cases with idiopathic CP, mutations in pancreatic serine protease inhibitor Kazal type 1 (SPINK1) have been detected. Although these findings may explain development of CP in some patients, the pathogenesis in the majority of cases remains unknown, and it is likely that there are additional genetic factors modifying the susceptibility towards CP [1,3].

The mechanism of tissue damage in CP is unclear.

Various toxins have been implied as a possible cause, including oxidative stress from endogenous origin or chemical stress by environmental or lifestyle related xenobiotics [7].

UDP-glucuronosyltransferases (UGTs), a family of biotransformation enzymes located in the endoplasmic reticulum of many cell types, contribute to the detoxification of a number of common, potentially harmful chemicals, by catalyzing the addition of the glucuronyl group from UDP-glucuronic acid, to a wide variety of endogenous or exogenous compounds [8]. The resulting metabolites in general are less biologically active and possess increased water solubility, enhancing their biliary or renal excretion. Beside detoxification however, biotransformation reactions catalyzed by UGT enzymes in a minority of cases, can result in bioactivation of molecules. Reactive intermediates and toxic metabolites can be liberated with detrimental consequences [9–11].

UGT enzymes have been classified on the basis of sequence homology into the UGT1A, UGT2A and 2B

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subfamilies [12]. Family 1A isoforms are derived from a single gene locus on chromosome 2q37 [13]. In humans the UGT1A is composed of at least nine functional proteins (UGT1A1, UGT1A3–UGT1A10), encoded by five exons. Exons 2 to 5 are shared by all UGT1A genes, whereas exon 1 is different and determines the specific properties of the UGT1A members [12]. Family 2 enzymes include UGT2A1, 2B4, 2B7, 2B10, 2B11, 2B15 and 2B17, encoded by separate genes, mapped on chromosome 4 [14]. Most human UGT isoforms are expressed in the liver, but high UGT expression levels have also been reported extrahepatically, indicating that other organs also play a major role in glucuronidation [15–17].

Recently, functional polymorphisms in the genes encoding the various UGT1 family members have been discovered [18–21]. UGT1A1 has been most extensively studied due to its importance in clearing bilirubin. So far over 100 different substrates have been identified, including endogenous estrogens and numerous xenobiotic compounds, such as phenols and flavonoids [22–24]. A genetic polymorphism in the promotor region of *UGT1A1*, which contains an extra TA repeat $(TA)_7TAA$ instead of $(TA)_6TAA$, results in reduced gene expression with concomitant reduction in enzyme activity [25–27]. This so-called TATA box mutation (*UGT1A1*28*) in Caucasians may lead to hyperbilirubinemia and is associated with Gilbert's syndrome [28,29].

UGT1A6 has the capacity to glucuronidate many xenobiotic phenols and genetic defects may lead to lower rates of metabolism of a number of phenols [30]. Two missense mutations on the same allele of *UGT1A6 (UGT1A6*2)* result in T181A and R184S amino acid substitutions with reduced enzyme activity [30,31]. In most cases both mutations are linked, although the single R184S mutation has been identified incidentally. The presence of an *UGT1A6*2* allele may reduce the protective effect of aspirin against colon adenomas [32].

UGT1A8 in the gastrointestinal tract is expressed exclusively extrahepatic, and might play an important role in first pass metabolism [18]. The UGT1A8 enzyme participates in the metabolism of drugs, dietary and environmental carcinogens in addition to endogenous substrates [33]. Recently, three allelic variants of the *UGT1A8* have been identified: *UGT1A8*1, UGT1A8*2* and *UGT1A8*3*, resulting from two amino acid substitutions at positions 173 and 277. Both *UGT1A8*1* and *UGT1A8*2* gene products have similar catalytic properties, whereas presence of the *UGT1A8*3* allele leads to dramatically reduced activity towards substrates [20].

Recently, genetic polymorphisms in another extrahepatic member of the UGT family (UGT1A7) was possibly linked to an increased risk for CP and pancreatic cancer [34]. We therefore hypothesized that toxins, either of endogenous or exogenous origin and potential substrates for UGT1A1, 1A6 or 1A8, could be also involved as causative agents for CP. Since genetic variations in enzymes metabolizing such molecules could also modify the risk for CP, we investigated the possible association between CP and genetic polymorphisms in *UGT1A1*, *UGT1A6* and *UGT1A8*, in a cohort of nonselected Caucasian CP patients.

Methods
Subjects
The study was approved by the local medical ethical review committee and all subjects gave their written informed consent. All subjects studied were Caucasians of Dutch (50%) and Hungarian (50%) extraction. The CP patients group consisted of 258 adult patients (164 males, 94 females). Twenty-five patients (10%) had a family history of CP (HCP), 153 patients (59%) had alcohol-induced CP (ACP) and 80 patients (31%) were classified as having idiopathic chronic pancreatitis (ICP).

The clinical diagnosis of CP was based on one or more of the following criteria: presence of typical complaints (recurrent upper abdominal pain, radiating to the back, relieved by leaning forward or sitting upright and increased after eating); suggestive radiological findings, such as pancreatic calcifications or pseudocysts, and pathological findings (pancreatic ductal irregularities and dilatations) revealed by endoscopic retrograde pancreaticography or magnetic resonance imaging of the pancreas before and after stimulation with secretin. HCP was diagnosed on the basis of two first-degree relatives or three or more second-degree relatives in two or more generations, which suffered from recurrent acute pancreatitis or chronic pancreatitis for which there was no precipitating factor. ACP was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than 2 years. Four of the 153 ACP patients also suffer from liver cirrhosis. Patients were classified as having ICP when precipitating factors such as alcohol abuse, trauma, medication, infection, metabolic disorders and/or a positive family history were all absent.

For comparison, we collected a control group consisting of 140 adult healthy subjects (53 males, 87 females) recruited by advertisement in a local paper.

Blood
For DNA extraction, blood samples were collected into EDTA tubes and stored at −20°C until use. DNA was isolated from whole blood using the Pure Gene DNA isolation kit according to the instructions from the

manufacturer (Gentra Systems, Minneapolis, MN, USA), diluted to 50 mg/ml and stored at 4°C.

Genotyping

In the *UGT1A1* gene, the number of TA-repeats in the promotor region was analyzed using the PCR conditions and primers (C and D) as described before [26]. Amplification was confirmed by agarose electrophoresis before fragments were resolved on 13% polyacrylamide gels (19:1 acrylamide/bisacrylamide; Biorad) in Tris-borate EDTA buffer as described before [35]. Gels (20 × 20 × 0.075 cm) were run at 400 V for 4 h and stained with ethidium bromide for 15 min. Fragments of 98 bp indicate for the *UGT1A1*1* allele, containing six TA repeats, and fragments of 100 bp indicate for the *UGT1A1*28* allele, containing seven TA repeats.

The T181A and R184S polymorphisms in exon 1 of the *UGT1A6* gene were studied using polymerase chain reaction amplification according to the method recently described [36]. Subsequently the PCR product was digested using restriction enzymes *Nsi*I for the T181A substitution and *Fnu*4HI for the R184S substitution. Digested samples were run on a 3% agarose gel and stained with ethidium bromide. The *UGT1A6*2* allele contains an additional restriction site for both restriction enzymes used here, which is not present in the *UGT1A6*1* allele.

Both polymorphisms in the *UGT1A8* gene corresponding with amino acid substitutions at position 173 and 277 were analyzed with two separate PCRs followed by restriction fragment length polymorphism analysis. The forward and reverse primers used for the PCR to detect the A173G substitution were 5′-CAGTTCTCTCATG GCTCGCA-3′ and 5′-GTGTGGCTGTAGAGATCAT ATGCT-3′, respectively. The PCR conditions were 4 min at 95°C, then 35 cycles of 30 s at 95°C, 1 min at 58°C, and 1 min at 72°C, and finally an elongation step at 72°C for 7 min. A 750 bp product was amplified and aliquots of 5 μl of the PCR mixture were directly digested for one hour at 37°C with 10 units of the restriction enzyme *Alu*I, followed by electrophoresis on 3% agarose gel, containing ethidium bromide. The *UGT1A8*2* allele (A173G) contains only one restriction site for *Alu*I, instead of two restriction sites for the *UGT1A8*1* and *UGT1A8*3* alleles (Fig. 1a). To detect the C277Y substitution by PCR, the forward and reverse primers 5′-TCTTCATTGGTGGTATCAGCT-3′ and 5′-AAAATTTGATAACTGATGAGTACATA-3′ were used, respectively. The annealing temperature for this PCR was 1 min 49°C, with remaining similar PCR conditions. The last G of the forward primer creates a restriction site for *Pvu*II; the digestion of the 215 bp PCR product with *Pvu*II was carried out under similar conditions as described above. The *UGT1A8*3* allele (C277Y) contains no restriction site for *Pvu*II, distinct from the *UGT1A8*1* and *UGT1A8*2* alleles, which have one *Pvu*II restriction site (Fig. 1b).

Statistical analyses

Data were analyzed by SAS version 8.0. Differences in the baseline characteristics of patients and controls were estimated with Fisher's exact test and the *t*-test. Chi-square statistics were used to estimate differences in the presence of allele frequencies in *UGT1A1*, *UGT1A6* and *UGT1A8* among the different study groups. When one of the genotypes has expected counts of less than 5, the Chi-square test may not be a valid test, and then we used Fisher's exact test. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated by logistic regression analysis for the allele frequencies in *UGT1A1* and for the *UGT1A8*3* compared to the *UGT1A8*1* and *UGT1A8*2* alleles taken together.

Fig. 1
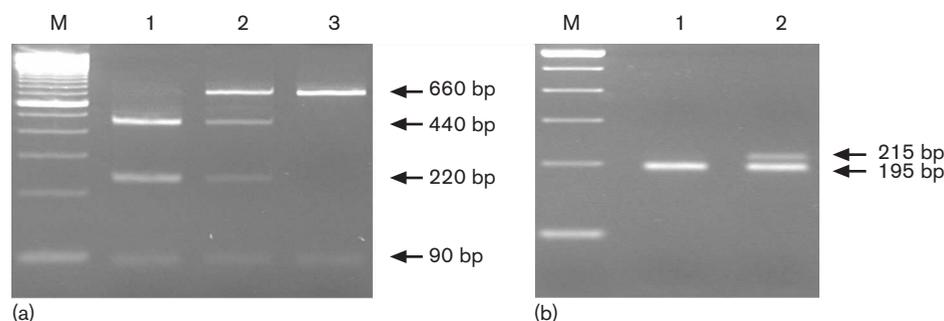
Identification of *UGT1A8* polymorphisms using restriction fragment length polymorphism analysis. Electrophoresis patterns of PCR fragments after digestion with *Alu*I for the A173G polymorphism (a) and after digestion with *Pvu*II for the C277Y polymorphism (b). M: 100 bp marker, lanes 1: homozygosity for the common allele, lanes 2: heterozygosity, lane 3: homozygosity for the variant allele. Homozygosity for the C277Y polymorphism was not found. The sizes of the PCR fragments are indicated by arrows.

The distribution of each allele frequency among the control population was tested whether it fitted the Hardy–Weinberg equilibrium.

In total, three genetic polymorphisms of the UGT1A family were analyzed. Because the different UGT1A isoforms are derived from one single gene locus, we corrected for multiple testing with Bonferroni, meaning that P values of less than 0.017 instead of 0.05 were considered to represent statistical significance.

Finally, we examined the co-occurrence of the alleles encoding less active UGT enzymes, UGT1A*28, UGT1A6*2 and UGT1A8*3 among CP patients and control subjects using the Spearman rank correlation test.

Results
Characteristics of patients and controls
Characteristics of patients with CP and healthy controls are denoted in Table 1. There is a difference in gender between patients and healthy controls, with more female subjects in the latter group ($P < 0.05$; Table 1).

The mean age of CP patients is higher compared to that of the control group ($P < 0.05$; Table 1), except for the HCP patients, since these patients develop CP at a younger age than the other CP patients.

Although data on smoking habits was only available from a limited number of subjects, significantly more ACP patients smoked compared to healthy controls ($P < 0.05$; Table 1).

UGT1A1, *UGT1A6* and *UGT1A8* polymorphisms
All UGT polymorphisms investigated are summarized in Table 2. Allele frequencies of the polymorphisms in the three *UGT1A* genes investigated here are reported in Table 3.

There were no statistical differences in the distribution of *UGT1A1*1* and *UGT1A1*28* alleles among patients

Table 2 Genetic polymorphisms in UGTs investigated here

Polymorphism	Allelic variation
UGT1A1*1	$(TA)_6TAA$
UGT1A1*28	$(TA)_7TAA$
UGT1A6*1	$T^{181}R^{184}$
UGT1A6*2	$A^{181}S^{184}$
UGT1A6 R184S	$T^{181}S^{184}$
UGT1A8*1	$A^{173}C^{277}$
UGT1A8*2	$G^{173}C^{277}$
UGT1A8*3	$A^{173}Y^{277}$

with HCP, ACP, ICP separately or in all CP patients combined vs. healthy control subjects (Table 3). We were unable to detect individuals carrying five or eight TA repeats in the TATA box of the promotor.

The distribution of the *UGT1A6*1*, *UGT1A6*2* and *UGT1A6 R184S* alleles in the different CP patient groups and control subjects does not demonstrate any statistical difference either (Table 3). There were no individuals with a single T181A variant, whereas approximately 2% of all subjects carried the single R184S variant.

UGT1A8 genotyping did not show a different distribution of the *UGT1A8*1*, *UGT1A8*2* and *UGT1A8*3* allele among patients compared to healthy control subjects (Table 3). Since *UGT1A8*1* and *UGT1A8*2* alleles may have similar catalytic properties, we combined these alleles, but this did not yield in significant differences between CP patients and controls. No statistical difference was observed in the frequencies of the *UGT1A8*3* allele (Table 3).

In addition, the co-occurrence of *UGT1A*28* and *UGT1A6*2* polymorphism were examined and strong association was found in both the CP patients and control group ($r = 0.66$, $P < 0.0001$ and $r = 0.78$, $P < 0.0001$, respectively). There was no statistically significant difference in the distribution of this combination of alleles between CP patients and healthy controls ($P = 0.58$). Because of the relatively low frequency of *UGT1A8*3* allele, the combination of all three alleles encoding for less active UGT enzymes was not examined.

For each of the three UGT1A genes the distribution of the allele frequencies among the control population was separately tested and found to fit with the assumption of the Hardy–Weinberg equilibrium.

Discussion
Genetic variations that may reduce expression or activity of phase II biotransformation enzymes are of toxicological and physiological importance and are frequently associated with a wide variety of diseases. Glucuronidation catalyzed by UGTs is one of the most

Table 1 Main characteristics of patients with chronic pancreatitis and healthy controls

Characteristic	HCP	ACP	ICP	All patients	Controls
n	25	153	80	258	140
Gender					
Male/female	9/16	117/36*	38/42	164/94*	53/87
Age (years) Mean ± SD	38 ± 21	51 ± 9*	48 ± 17*	49 ± 14*	44 ± 16
Smoking (yes/no)	8/6	91/7	31/11	130/24	68/45
Unknown	11	55	38	104	27

HCP: hereditary chronic pancreatitis; ACP: alcoholic chronic pancreatitis; ICP: idiopathic chronic pancreatitis.
*P-value < 0.05 as compared to control group.

Table 3 Allele frequencies of *UGT1A1*, *UGT1A6* and *UGT1A8* in patients with chronic pancreatitis and healthy controls

Alleles	HCP (n = 50)	ACP (n = 306)	ICP (n = 160)	All patients (n = 516)	Controls (n = 280)
UGT1A1					
*1	0.68	0.67	0.66	0.66	0.72
*28	0.32	0.33	0.34	0.34	0.28
P-value*	0.61	0.23	0.15	0.14	
OR (95% CI)	1.2 (0.6–2.3)	0.8 (0.6–1.5)	1.4 (0.9–1.8)	1.3 (0.9–1.8)	
UGT1A6					
*1	0.65	0.63	0.65	0.64	0.64
*2	0.30	0.34	0.34	0.34	0.34
R184S	0.04	0.03	0.01	0.02	0.02
P-value*	0.64	0.89	0.48	0.99	
UGT1A8					
*1	0.82	0.79	0.83	0.80	0.73
*2	0.16	0.20	0.15	0.18	0.25
*3	0.02	0.01	0.02	0.02	0.01
P-value*	0.35	0.33	0.04	0.06	
OR (95% CI)**	0.8 (0.2–12.9)	1.1 (0.3–4.4)	1.3 (0.3–6.0)	1.1 (0.3–3.7)	

HCP: hereditary chronic pancreatitis; ACP: alcoholic chronic pancreatitis; ICP: idiopathic chronic pancreatitis; OR: odds ratio; 95% CI: 95% confidence interval.
P-values, estimated with Chi-square test or with Fisher exact test, when cells have expected counts less than 5.
**OR 95% CI for the *UGT1A8*3* allele compared to the *UGT1A8*1* and *UGT1A8*2* alleles taken together.

important mechanisms involved in host defence against xenobiotic chemicals and endogenous toxins. Xenobiotically or endogenously mediated cellular injury might play a role in the etiology of CP [37–39]. In the present study we investigated the possible association between CP and genetic polymorphisms in three UGT1A isoenzymes that are associated with changes in enzyme activity and function and are potentially expressed in pancreatic tissue [20,25–27,30,31].

We found that frequencies of genetic polymorphisms in *UGT1A1*, *UGT1A6* and *UGT1A8* in Caucasian healthy control subjects were not different to those in CP patients. This suggests that individuals bearing the UGT alleles, encoding for less active enzymes do not have a higher risk for developing CP.

The frequency of the *UGT1A1*28* allele was 34% in CP patients compared to 28% in the controls. This is comparable to the frequencies (29–39%) reported in Caucasian control subjects by several other groups [27,30,40–42]. The frequency of the *UGT1A6*2* allele in both CP patients and healthy controls was estimated at 34%, which is appreciably higher than the 16.8% reported by Ciotti et al. [31], but not different from 30.7% and 32.5% found by Lampe et al. [30] and Köhle et al. [42], respectively. The allele frequency of *UGT1A8*3*, 1.6% for CP patients and 1.4% for healthy controls, was similar to 2.2%, reported by Huang et al. [20].

UGT1A7 is the only other member of the UGT1A family which has been recently investigated in relation to pancreatic diseases. Genetic polymorphisms possibly associated with a low detoxification activity of UGT1A7

have recently been identified by Ockenga et al. as a risk factor for alcohol-induced CP and pancreatic carcinoma [34]. They detected a much higher frequency of the *UGT1A7*3* allele in patients (25–37%) as compared to healthy controls (21%). As UGT1A7 catalyzes the glucuronidation of several tobacco smoke carcinogens, and smoking in addition to alcohol abuse, appears to be a risk factor for CP, reduced enzyme activity might confer a higher risk for CP. In addition, UGT1A7 is reported to be the predominant UGT1A transcript in human pancreas [34]. In contrast to Ockenga et al., who did find a positive association between the low detoxification allele *UGT1A7*3* and the risk for alcoholic chronic pancreatitis and pancreatic cancer, results from our laboratory indicate that individuals bearing less active *UGT1A7* alleles do not run a higher risk for pancreatic diseases (unpublished observations). We collected a large multi-national cohort of patients with either acute (n = 153) or CP (idiopathic/hereditary n = 266, alcoholic n = 318) or pancreatic cancer (n = 297) and compared the genetic polymorphisms in the *UGT1A7* gene with 1532 controls. Using melting curve analysis with fluorescence resonance energy transfer probes and restriction fragment length polymorphism analysis, we were unable to detect differences in UGT1A7 frequency distribution between patients and controls. Most notably, the frequency of the *UGT1A7*3* risk allele was comparable between patients (31–44%) and controls (40%). The discrepancies between our study and that of Ockenga et al. [34] most likely stem from differences in (a) selection of the control group, (b) sample size and (c) the different methodology used in both studies. The UGT isoenzymes UGT1A1 and UGT1A6, investigated in our study do not specifically glucuronidate benzo[a]pyrenes or other toxic com-

pounds from cigarettes [8], whereas UGT1A8 does [20]. However, it is not known yet whether the UGT1A8 enzyme is substantially expressed in pancreatic tissue.

UGTs are known to exist as a superfamily of enzymes with a broad substrate profile, however substrate specificity of the various isoenzymes remains poorly defined. Isoenzymes may exhibit overlapping substrate specificities, and one isoenzyme may compensate low activity of another, explaining why individuals bearing one low detoxification activity allele do not confer a higher risk for CP. Apparently the UGTs investigated here are not exclusively responsible for the metabolism of a particular drug or chemical associated with CP, or are not the predominant UGTs present in pancreatic tissue as might be for UGT1A7 in the pancreas, and therefore these polymorphisms did not show to be of major clinical significance.

Genetic polymorphisms in *UGT1A1* and *UGT1A6* are known to play important roles in disease susceptibility. Homozygosity or heterozygosity for genetic polymorphisms in *UGT1A1* may lead to hyperbilirubinemia (Crigler–Najjar or Gilbert's syndromes), because activity towards conjugation of bilirubin is restricted to UGT1A1 [17]. Nevertheless, the UGT1A1 promotor polymorphism, which is strongly linked to the UGT1A6 polymorphisms studied here [36], is reported to be positively associated with breast cancer in premenopausal African-American women, but not in Caucasians [40,43]. In addition, the *UGT1A6*2* allele is found to be associated with reduced protection against colon adenomas by aspirin [32].

In summary, we failed to detect associations between CP and genetic polymorphisms in *UGT1A1*, *UGT1A6* and *UGT1A8* in Caucasians. Our results might suggest that presence of low detoxification activity of these UGT enzymes does not predispose to CP.

Acknowledgements

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