

Pharmacogenetic analysis of adverse drug effect reveals genetic variant for susceptibility to liver toxicity

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ABSTRACT

A retrospective pharmacogenetic study was conducted to identify possible genetic susceptibility factors in patients in whom the administration of the anti-Parkinson drug, tolcapone (TASMAR[®]), was associated with hepatic toxicity. We studied 135 cases of patients with elevated liver transaminase levels (ELT) of ≥ 1.5 times above the upper limit of normal, in comparison with matched controls that had also received the drug but had not experienced ELT. DNA samples were genotyped for 30 previously described or newly characterized bi-allelic single nucleotide polymorphisms (SNPs), representing 12 candidate genes selected based on the known metabolic pathways involved in the tolcapone elimination. SNPs located within the UDP-glucuronosyl transferase 1A gene complex, which codes for the enzymes involved in the main elimination pathway of the drug, were found to be significantly associated with the occurrence of tolcapone-associated ELTs.

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INTRODUCTION

Tolcapone is a selective and potent inhibitor of catechol-O-methyltransferase (COMT), and is used to improve the bioavailability of L-dopa in the treatment of Parkinson's disease. A number of significant tolcapone-related liver injuries were reported during the first year after introduction of the drug,^{1–3} resulting in a marketing suspension in the European Union and stricter patient monitoring in North America and the rest of the world. Tolcapone-induced liver injuries are rare events: during the initial clinical trials none of the 3848 patients treated experienced clinical signs or symptoms indicative of serious drug-induced liver injury, but 1–3% of the patients did experience asymptomatic transient liver enzyme elevations. Animal toxicology studies failed to provide any insight into the pathomechanism of liver injury.^{4,5} In an attempt to shed additional light on this adverse event, we embarked on a retrospective clinical study to examine the potential role of genetic variants as predisposing factors to tolcapone-induced liver injury.

The main metabolic routes for tolcapone metabolism⁶ are summarized in Figure 1, and genetic variations in these enzymes were analyzed with regard to their prevalence in ELT cases and controls, respectively. In addition, we investigated whether genetic variations in enzymes involved in oxidative stress

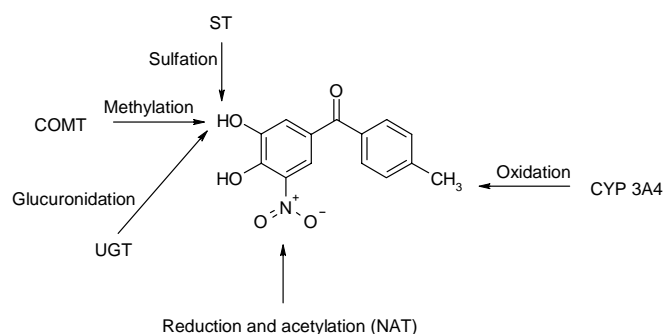


Figure 1 Primary metabolic routes of tolcapone metabolism (ST = sulfotransferase; NAT = N-acetyltransferase; COMT = catechol-O-methyl transferase; UGT = UDP-glucuronosyltransferase; CYP3A4 = cytochrome P450 3A4).

response, mitochondrial uncoupling and glutathione-S-transfer (Table 1) might show associations with tolcapone-induced liver toxicity.

RESULTS

The study was designed to include patients who had received tolcapone in previous clinical trials. The cases in this study ($n = 135$) were Parkinson’s patients who, while being treated with tolcapone, demonstrated ELT of $1.5 \times$ the upper limit of normal (ULN) or more. The controls ($n = 274$) were Parkinson’s patients who showed no signs of ELT while treated with tolcapone. Patient data were categorized by case–control status and by genotype at any of the markers tested. First-pass analysis revealed statistically significant associations between prevalence of ELT and homozygosity for the G allele at marker *UGT1A6*-A528G (OR = 2.76, 95% CI = 1.5–5.06; $P < 0.001$) as well as nine other markers in the UDP-glucuronosyltransferase gene complex, all of which are in significant linkage disequilibrium with *UGT1A6*-A528G (see Table 1). Stratification of the sample according to sex revealed that the observed association between ELT and *UGT1A6*-A528G genotype was limited exclusively to male patients (OR 5.18; 95% CI = 2.37–11.34; $P < 0.0001$) and was not observed in females (OR = 1.08, 95% CI = 0.38–3.00; NS).

Table 1 Summary of the genetic markers analyzed

Gene	SNP	Amino acid change	P-value	Accession number
CAT	G1169A	Gly389Asp	n.s.	E01497
COMT	G293T	Ala72Ser	n.s.	M65212
COMT	A551G	Met158Val	n.s.	M65212
CYP3A4	A729G	Promoter region	n.s.	AF181105
GSTM3	del AGG4598	Intron	n.s.	AF043105
GSTP1	A331G	Ile105Val	n.s.	X15480
ALB	G1987T	Lys313Asn	n.s.	V00495
SOD2	C90T	Ala16Val	n.s.	E01408
SOD2	T288C	Ile82Thr	n.s.	E01408
NAT1	G560A	Arg187Gln	n.s.	AF008204
NAT1	C190T	Arg64Trp	n.s.	AF008204
NAT2	C1063T	Ile114Thr	n.s.	X14672
NAT2	G1312A	Arg197Gln	n.s.	X14672
NAT2	G1578A	Gly286Glu	n.s.	X14672
SULT1A1	G1112A	Arg213His	n.s.	XM_012525
SULT1A1	A1141G	Met223Val	n.s.	XM_012525
UCP2	C164T	Ala55Val	n.s.	U76367
UGT1A	C908G*	3'-UTR region	0.0023	M84124
UGT1A	G473T*	Gly/Val _(exon4)	n.s.	M84123
UGT1A6	T232G	Ser7Ala	0.0106	M84130
UGT1A6	A528G*	Silent	0.0008	M84130
UGT1A6	A754G	Thr181Ala	0.0018	M84130
UGT1A6	A765C	Arg184Ser	0.0023	M84130
UGT1A7	A197C*	Silent	0.0235	U39570
UGT1A7	G551T	Asn129Lys	0.0494	U39570
UGT1A7	A555C	Arg131Lys	0.0494	U39570
UGT1A7	A556G	Arg131Lys	0.0494	U39570
UGT1A7	T786C	Trp208Arg	0.0252	U39570
UGT1A7	G920A*	Silent	n.s.	U39570
UGT1A8	C245A*	Promoter	n.s.	U42604

*SNPs that were discovered in this study. The UGT1A markers listed here correspond to different splice variants that arise from the use of different promoters and first exons of the UGT1A gene (see Figure 3). Thus, for example, the UGT1A6 SNPs refer to those found in the first exon of the UGT1A6 transcript.

We determined pairwise strength of association between SNPs in the *UGT1A* gene. Figure 2 shows results for all pairs of the 13 SNPs in this gene for the case population. An analogous figure for the control population is not presented here because many SNPs were monomorphic. Clearly, except for SNPs located near the 5' or 3' ends of the gene (*UGT1A8*-C245A, *UGT1A*-G473T and 3'UTR-C908G), all were in complete disequilibrium with one another. Haplotypes for the 13 SNPs in the *UGT1A* gene were determined by Clark's algorithm,⁷ which does not require the assumption of Hardy–Weinberg equilibrium. Based on the genotypic results, a total of 44 haplotypes were inferred from 818 chromosomes and were assigned to each individual. Fifteen of the 44 haplotypes had frequency counts ≥ 5 in both case and control groups, accounting for approximately 93% of the total (Table 2). In these 15 haplotypes, two single sites (*UGT1A8*-C245A and *UGT1A*-G473T) are monomorphic. For one region, three consecutive SNPs (*UGT1A7*-G551T, *UGT1A7*-A555C and *UGT1A7*-A556G) are in complete linkage disequilibrium, that is, only two haplotypes (G–A–A and T–C–G) occur. Association between these 15 haplotypes and case–control status was statistically highly significant ($P = 0.0027$). Haplotype 1, cAGAACgGGGCgC, and haplotype 4, cCTCGTgTAAAgG, render the highest statistical effect on differentiating cases from controls—removing either one of them decreases the statistical significance (the P -value increases about 10-fold), while

removing other haplotypes had only slight effects (Table 2). For the two haplotypes, three sites were monomorphic (same lowercase nucleotides). At the remaining sites the two haplotypes show different alleles (uppercase nucleotides). Haplotype 1 is associated with disease (more frequent in cases than expected) while haplotype 4 is more common in control individuals than expected by chance.

To analyze the data further, principal components analysis was carried out among the 10 SNPs that showed P -values of 0.05 or less (Table 1), resulting in the identification of two principal components, P1 and P2, which accounted for 97% of the genotypic variation among the 10 markers (for case and control individuals combined). Multivariate regression analysis for P1, P2, and sex revealed that these three variables account for 21% of ELT variance ($P < 0.0001$). The power of the marker genotypes was sufficient to allow accurate prediction in 61% of ELT status.

DISCUSSION

Given that glucuronidation is the main pathway for tolcapone elimination (Figure 1), our findings are consistent with the hypothesis that impaired elimination of tolcapone may contribute causally to the observed liver toxicity. This interpretation is further supported by the finding that the *UGT1A6* Ala181/Ser184 variant—which arises from the

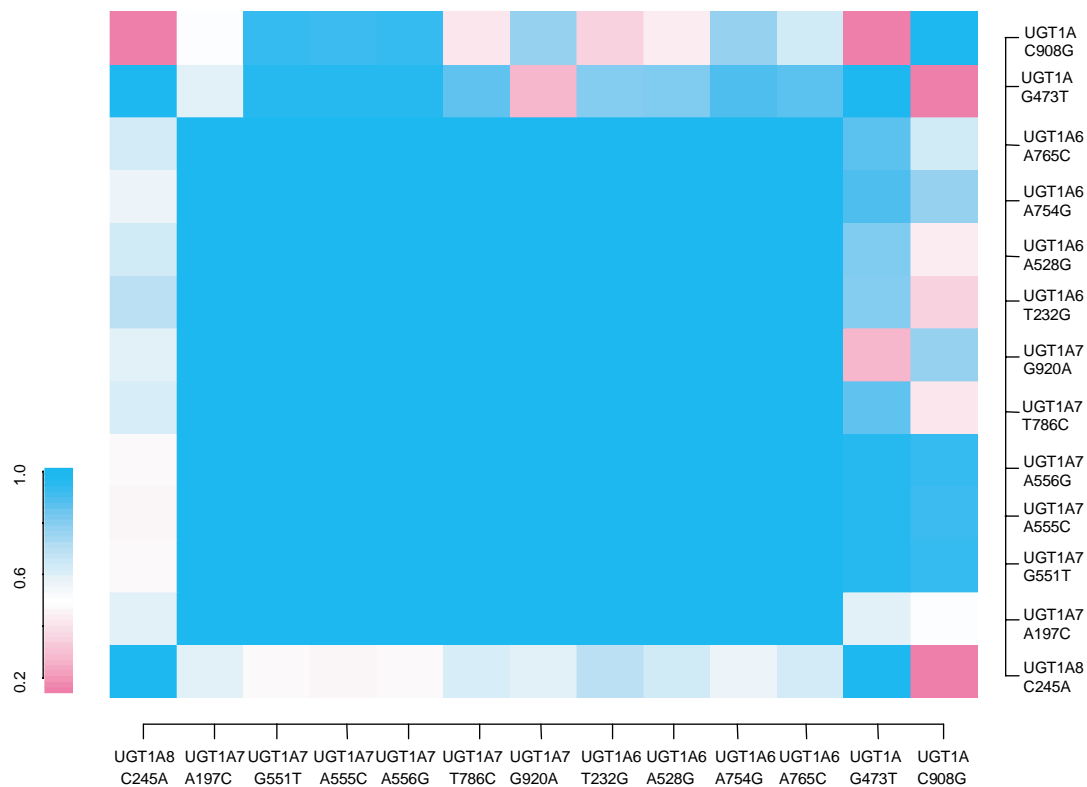


Figure 2 Representation of the pairwise association strength between the 13 SNPs in the *UGT1A* gene of the case population. The color gradient from blue to red represents strong (blue) to weak (red) association.

Table 2 Haplotype frequencies

HAP	UGT1 Site													Frequency (%)					
	A8	A7	A7	A7	A7	A7	A7	A6	A6	A6	A6	A1	3'UTR	Case	Control	Observed	Expected	Observed	Expected
1	C	A	G	A	A	C	G	G	G	G	C	G	C	7.4	5.9	10.8	12.2		
2	C	C	G	A	A	T	A	T	A	A	A	G	C	3.9	3.9	8.0	8.0		
3	C	C	T	C	G	T	G	T	A	A	A	G	C	6.0	6.3	13.3	12.9		
4	C	C	T	C	G	T	G	T	A	A	A	G	G	1.6	2.6	6.2	5.3		
5	C	A	G	A	A	C	G	G	A	A	A	G	C	0.5	0.8	2.0	1.6		
6	C	C	G	A	A	T	A	T	A	A	A	G	G	1.2	1.7	3.8	3.4		
7	C	C	T	C	G	C	G	T	A	A	A	G	C	2.0	1.6	2.9	3.3		
8	C	C	G	A	A	C	A	T	A	A	A	G	G	0.4	0.5	1.2	1.1		
9	C	C	G	A	A	C	A	T	A	A	A	G	C	1.2	0.8	1.4	1.7		
10	C	A	G	A	A	C	G	G	G	G	C	G	G	1.2	1.4	3.2	2.9		
11	C	A	G	A	A	T	G	G	G	G	C	G	C	3.5	2.9	5.2	5.9		
12	C	A	T	C	G	C	G	G	G	G	C	G	C	0.5	0.6	1.5	1.3		
13	C	C	G	A	A	T	G	T	A	A	A	G	G	0.4	0.6	1.6	1.3		
14	C	C	G	A	A	T	G	G	G	A	C	G	C	0.5	0.5	1.1	1.1		
15	C	C	G	A	A	T	G	G	G	A	A	G	C	0.4	0.4	0.9	0.9		
R														5.7		4.1			

R indicates all the remaining haplotypes. Chi-square test for the association between cases and the 15 haplotypes received *P*-value 0.0027. Bold indicates the corresponding haplotypes are most crucial for association to the cases.

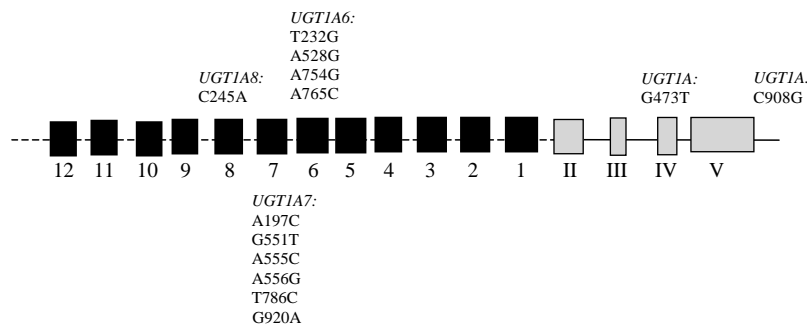


Figure 3 The *UGT1A* gene consists of at least 9 promoters and first exons (indicated as black boxes; first exons 3, 11 and 12 are pseudogenes) which can be spliced with 4 common exons (gray boxes, indicated by roman numerals) to result in 9 different *UGT1A* enzymes. The relative position of the SNPs tested in this gene is indicated. The nucleotide position of the SNPs corresponds to their position within the indicated exon sequence (see Table 1 for the sequence annotation).

UGT1A6 G754/C765 haplotype—has previously been found to show reduced enzymatic activity *in vitro* compared with the wild type⁸ and might thus be associated with impaired clearance of the drug. This concurs with the findings from the current analysis, in which the presence of the Ala181 and Ser184 variants was associated with incrementally higher risks for ELT. For the polymorphism located in the 3'UTR of *UGT1A* the less frequent allele appears to have a protective effect, as it is more frequent in control patients than in patients with ELT. Since the *UGT1A* genes potentially share the 3' regulatory region, this polymorphism may affect the expression of several or all *UGT1A* genes⁹ involved in the metabolism of tolcapone (Figure 3). Alternatively, this

polymorphism may be in linkage disequilibrium with another polymorphism that affects either the structure of the *UGT1A* proteins or the expression of the gene. The observation that the *UGT1A* polymorphisms are only significantly associated with ELTs in male but not in female patients could result from the lower number of females in the study, or it may suggest that the mechanism leading to tolcapone-induced ELT is different in males and females. Finally, no significant association was found between ELT and any of the other genetic variants tested (Table 1).

ELT is a well-established, yet indirect, indicator of liver damage. The relatively low cut-off value of 1.5 times above the ULN was selected to increase sensitivity of the study. As a

consequence, the study population is perhaps more likely to include patients whose ELT was the product of factors other than tolcapone than had been the case with a higher cut-off value.

The relatively modest odds ratios found for association between ELT and individual marker genotypes are consistent with the concept that drug-induced liver toxicity represents a complex, multifactorial phenomenon. Thus, in addition to heritable predisposition (and the role of sex) presented here, additional factors—such as additional genes not considered here, and/or extrinsic influences (such as co-medication, nutrition, etc) as well as complex interactions among genetic and environmental factors—are all likely to contribute to the ELT phenotype. The limited positive predictive power of the risk genotype identified makes it an unlikely candidate for a clinically useful diagnostic tool for predicting patients at risk of tolcapone-induced liver toxicity. Nevertheless, the identification of a susceptibility gene/haplotype represents a first step towards the identification of a mechanism contributing or causing toxicity in humans. We believe that this study, therefore, represents an illustrative and pragmatic real-world example for what we may commonly expect to find in similar situations, with regard to the promise, but also the limitations of pharmaco- and toxicogenetic approaches.

METHODS

Patient Selection

Initially, 645 patients who had received tolcapone in previous clinical trials were considered for inclusion in this retrospective genetic analysis. This included 215 patients (cases) who had developed ELT, and 430 patients matched by age (within ± 10 years), and study protocol (which provided matching for disease severity). Of the 215 ELT patients, 135 agreed to participate in the study; another 31 patients did not participate because local Institutional Review Boards failed to approve the protocol (mainly due to local regulation or sensitivities concerning genetic research); and 49 patients were either lost to follow-up or deceased. Accordingly, 274 of the controls were retained to provide a 1 : 2 ratio. All cases and controls were of European origin and included a total of 261 males (74 cases, 187 controls) and 148 females (61 cases, 87 controls). Participation in this study was voluntary, and the patients' data were anonymized. All participating patients signed an appropriate informed consent before donating 9 ml of whole blood for DNA analysis.

ELT was classified as at least one measurement above 1.5 times the ULN of the investigator's range for aspartate aminotransferase or alanine aminotransferase. The ULN range was used instead of the absolute values to avoid the variation resulting from the different measurement procedures applied by each investigator in the different centers.

SNP Discovery in the *UGT1* Gene Complex

All exons of the *UGT1* locus (Figure 3) were sequenced in DNA samples derived from 47 non-related individuals of different ethnic origin (obtained from Coriell Institute of

Medical Research in Camden, NJ, USA). DNA sequencing and SNP identification were carried out as described.¹⁰ SNPs thus newly identified are annotated by an asterisk in Table 1.

Genotyping

DNA was extracted from 400 μ l whole blood using a silica gel-based extraction method (QiaAmp DNA Blood Kit, Valencia, CA, USA). Samples were genotyped using either automated fluorescent dideoxytermination-based DNA sequencing or the kinetic thermocycling method (KTC).¹¹ The primers used for PCR amplification and sequencing are listed in Table 3.

In the KTC format, the generation of double-stranded amplification product is monitored using a DNA intercalating dye and a thermal cycler which has a fluorescence-detecting CCD camera attached (PE-Biosystems GeneAmp 5700 Sequence Detection System). Fluorescence in each well of the PCR amplification plate is measured at each cycle of annealing and denaturation. The cycle at which the relative fluorescence reached a threshold of 0.5 using the SDS software from PE-Biosystems was defined as C_t . The amplification reactions were designed to be allele-specific, so that the amplification reaction was positive if polymorphism was present and negative if polymorphism was absent. For each bi-allelic polymorphism, one well of the amplification plate was set up to be specific for allele 1 and a second well was set up to be specific for allele 2. For each polymorphism to be detected, three primers were designed—two allele-specific primers and one common primer (Table 4). The amplification conditions were as follows: 10 mM Tris pH 8.0, 40 mM KCl, 2 mM MgCl₂, 50 μ M each of dATP, dCTP and dGTP, 25 μ M of TTP and 75 μ M of dUTP, 4% DMSO, 0.2X SyBr Green (Molecular Probes, Eugene, OR, USA), 2% glycerol, uracil *N*-glycosylase (UNG, 2 units), Stoffel Gold DNA polymerase (15 units, obtained from D. Birch, Core Research Department, RMS)¹² and primers (0.4 μ M) in an 85 μ l volume for each well. Thirty nanograms of DNA in a 15 μ l volume was then added to each well. To

Table 3 PCR and sequencing primers for genotyping the *UGT1A* gene

Gene/exon no.	Type	Primer sequence 5' → 3'
<i>UGT1A6</i> /exon 1 (fgmt1)	Fwd	ACACGGCCATAGTTGGTTCA
	Rev	CAGTTGATGAAGTACAGGCC
<i>UGT1A6</i> /exon 1 (fgmt2)	Fwd	TGTAGTGGTGGTGCCTGAAG
	Rev	GACAGCTGATGCCGAGTTCTTC
<i>UGT1A7</i> /exon 1 (fgmt1)	Fwd	GAGGGCAGGTTCTATCGTAC
	Rev	GGGCACTGTGCACCTTCTTC
<i>UGT1A7</i> /exon 1 (fgmt2)	Fwd	ACGGCACCATTGCGAAGTGC
	Rev	ACTTACATATCAACAAGTGCTGC
<i>UGT1A8</i> /exon 1	Fwd	GGGCATGATCTGTCCAAGGC
	Rev	GGTTGAGTAAGTCTTCACTGTG
<i>UGT1A</i> /exon 4	Fwd	GGCCAACATATCCTACATTG
	Rev	CGTATTAATGCTACGTAAATGT
<i>UGT1A</i> /3'UTR	Fwd	CGTGCTGACAGTGGCCTTC
	Rev	CAGTGCCTCCAAGCCATTC

Table 4 Primers used to genotype markers by kinetic thermocycling

Marker	Primer type	Primer sequence (5' → 3')	Annealing temperature
ALB K313N	AS1	CATAGTTTTGCAAACATCC	56
	AS2	CATAGTTTTGCAAACATCA	56
	Common	TCATTAGCTGCTGATTTTGT	56
CAT G389D	AS1	CCTGCATGCACATCGGGC	58
	AS2	CCTGCATGCACATCGGGT	58
	Common	TACCTGTGAAGTGTCCCTAC	58
COMT S72A	AS1	ATGCGGAGCCCGGAACT	58
	AS2	ATGCGGAGCCCGGAAACG	58
	Common	CTTCTGCTCGCAGTAGGT	58
COMT V158M	AS1	GCACACCTTGCCTTCAT	58
	AS2	GCACACCTTGCCTTCAC	58
	Common	CATCACCATCGAGATCAAC	58
CYP3A4 A/G	AS1	CTATTAAATCGCCTCTCTCT	56
	AS2	CTATTAAATCGCCTCTCTCC	56
	Common	GGATGAATTTCAAGTATTT	56
GSTM3 GSTM3*A/B	AS1	CTGCAGAGATAGAGAAGTATCC	56
	AS2	CCTGCAGAGATAGAGAAGTATCT	56
	Common	AAAGGTAGGAAGAAGGGAAA	56
GSTP1 I105V	AS1	TTGGTGTAGATGAGGGAGAT	58
	AS2	TTGGTGTAGATGAGGGAGAC	58
	Common	ATGACGGCGTGGAGGA	58
MnSOD V16A	AS1	AGCCCAGATACCCCCAAAG	58
	AS2	AGCCCAGATACCCCCAAA	58
	Common	TGTGCTTTCTCGTCTTCA	58
MnSOD I82T	AS1	GAGATGTTACAGCCCAGAT	58
	AS2	GAGATGTTACAGCCCAGAC	58
	Common	ATGATTGATATGACCACCAC	58
NAT1 R64W	AS1	GATCAAGTTGTGAGAAGAAATC	58
	AS2	TGATCAAGTTGTGAGAAGAAATT	58
	Common	TTGACCTGGAGACACCAT	58
NAT1 R187Q	AS1	CTAGAAGACAGCAAATACCG	58
	AS2	CCTAGAAGACAGCAAATACCA	58
	Common	AATTGTTGAGGCTTAAGAG	58
NAT2 I114T	AS1	TGTAATTCCTGCCGTCAG	58
	AS2	TGTAATTCCTGCCGTCAG	58
	Common	ATACAGCACTGGCATGG	58
NAT2 R197Q	AS1	GACTCAAATCTTCAATTGTTT	56
	AS2	GACTCAAATCTTCAATTGTTT	56
	Common	AAATATACTTATTACGCTTGAAC	56
NAT2 G286E	AS1	TCCTTATTCTAAATAGTAAGGGATC	54
	AS2	TCCTTATTCTAAATAGTAAGGGATT	54
	Common	ATTCCTTGGGGAGAAAT	54
SULT1A1 M223V	AS1	AGGAGACCGTGGACTTCA	58
	AS2	AGGAGACCGTGGACTTCC	58
	Common	CTTGAACGACGTGTGCTG	58

Table 4 (continued)

Marker	Primer type	Primer sequence (5' → 3')	Annealing temperature
SULT1A1 R213H	AS1	CCTGGAGTTTGTGGGGCG	58
	AS2	CCTGGAGTTTGTGGGGCA	58
	Common	TGAACCATGAAGTCCACG	58
UCP2 A55V	AS1	GCCAGTGC GCGCTACAGC	58
	AS2	GCCAGTGC GCGCTACAGT	58
	Common	ACCATGGTCAGAATGGTG	58
UGT1A6 T181S	AS1	CGTGTTCCCTGGAGCATA	58
	AS2	CGTGTTCCCTGGAGCATG	58
	Common	GAATGTAGGACACAGGGTCT	58
UGT1A6 R184S	AS1	GACACAGGGTCTGGGCTT	58
	AS2	GACACAGGGTCTGGGCTG	58
	Common	TACCTTTCAGGGGTTTTTC	58
UGT1A C908G	AS1	TGCAGTAGGGGCAGCG	58
	AS2	TGCAGTAGGGGCAGCC	58
	Common	GGAGTGC GGGGATTCAA	58

reduce the possibility of contamination by pre-existing amplification product, the assay procedure included the incorporation of dUTP into the amplification product and an incubation step with uracyl *N*-glycosylase (UNG) for degradation of pre-existing uracyl-containing products.¹³ The thermal cycling conditions were as follows: 5 min at 50°C for UNG degradation of any previously contaminating PCR products, 12 min at 95°C for Stoffel Gold polymerase activation, 55 cycles of denaturation at 95°C and annealing at the annealing temperature indicated in Table 4, followed by a dissociation step of 1 min at 1° increments from 60°C to 95°C. The C_t of each amplification reaction was determined and the difference between the C_t for allele 1 and allele 2 (ΔC_t) was used as the assay result. Samples with ΔC_t 's between -3.0 and 3.0 were considered heterozygous, and samples with C_t 's below -3.0 or above 3.0 were considered homozygous for allele 1 or 2, respectively.

Statistical Analysis

Patient samples were stratified by case-control status and by genotype at any of the markers tested. Data were analyzed by the logistic regression analysis using recessive and dominant models. Odd ratios and 95% confidence intervals that excluded unity were regarded as statistically significant. This analysis was subsequently repeated after stratification by sex.

Multivariate Analysis and Interaction Modeling

A two-stage approach,¹⁴ with marker selection followed by a modeling stage, was employed, by first selecting all markers that were associated with case-control status at $P < 0.05$ in the chi-square test for comparing genotype frequencies between cases and controls (Table 1).

Of these, the best linear combinations were obtained by principal components analysis and used, along with sex, and predictive variables for multivariate logistic regression analysis. The estimated logistic regression equation reads as follows: $\log(\text{odds}) = 0.46(P1) + 1.36(P2) + 0.40(\text{sex}) - 0.29(P1 \times S) - 0.71(P2 \times S)$, where $P1$ and $P2$ are the main components, and S equals 1 for male and 2 for female (ie, the $\log(\text{odds})$ is predicted differently for males and females). The corresponding sex-specific equations are

$$\log(\text{odds}) = 0.17(P1) + 0.65(P2) + 0.40 \quad \text{for males}$$

$$\log(\text{odds}) = -0.12(P1) - 0.06(P2) + 0.80 \quad \text{for females.}$$

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DUALITY OF INTEREST

None declared.

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