

Sequence variations in the UDP-glucuronosyltransferase 2B7 (*UGT2B7*) gene: identification of 10 novel single nucleotide polymorphisms (SNPs) and analysis of their relevance to morphine glucuronidation in cancer patients

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ABSTRACT

We have screened a cohort of 239 Norwegian cancer patients for sequence variation in the coding and regulatory regions of the UDP-glucuronosyltransferase 2B7 gene (*UGT2B7*) and analyzed the impact of gene variants on morphine glucuronidation *in vivo*. In all, 12 single nucleotide polymorphisms (SNPs) were identified, 10 of which have not been previously described. Only one SNP causes a change in amino acid sequence (H268Y). Seven *UGT2B7* genotypes were observed and three main haplotypes predicted. There was no correlation between *UGT2B7* genotype or haplotype and morphine glucuronide to morphine serum ratios among 175 patients who received chronic oral morphine therapy, and who had normal renal and hepatic function. The apparent lack of functional polymorphisms fits well with the near unimodal, but broad, distributions of the ratios (morphine 3-glucuronide/morphine: 6.4–309.2; morphine 6-glucuronide/morphine: 0.5–72.8). Our results suggest that factors other than *UGT2B7* polymorphism may be more deciding for the variability in morphine glucuronide to morphine serum ratios.

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INTRODUCTION

The metabolic process of glucuronidation converts numerous endobiotics and xenobiotics to more hydrophilic metabolites. The process facilitates the substrate detoxication and excretion, and is catalyzed by UDP-glucuronosyltransferases (UGTs). UGTs are glycoproteins, localized in endoplasmatic reticulum (ER) and nuclear membranes. One important member of the UGTs is the *UGT2B7* isoform, which participates in the glucuronidation of several physiologically important endogenous compounds such as steroid hormones, bile acids, retinoids and fatty acids. This isoform also metabolizes drugs such as opioids and numerous carboxylic acid-containing drugs including the nonsteroidal anti-inflammatory drug (NSAID)-clofibric acid, and the anticonvulsant valproic acid.^{1,2} The

glucuronidation of morphine and codeine³ as well as zidovudine (AZT),⁴ a therapeutic agent used in HIV treatment, is exclusively catalyzed by UGT2B7. Nondrug xenobiotic substrates include hydroxylated derivatives of the prototypic carcinogens 2-acetylaminofluorene and benzo[*a*]pyrene.⁵ Although UGT2B7 is, in general, involved in the detoxication of endogenous and xenobiotic substrates, it also plays a critical role in the generation of bioactive or even toxic compounds such as the highly cholestatic D-ring glucuronides of estrogens,⁶ and the acyl-glucuronides of drugs such as diflunisal, which binds to proteins and elicits toxic immunological responses.⁷

UGT2B7 is predominantly expressed in the liver, but tissue distribution analysis has also demonstrated expression in the gastrointestinal (GI) tract, kidney, pancreas and brain.⁸ The *UGT2B7* gene is localized within a cluster of *UGT2B* genes at chromosomal position 4q13. The gene contains six exons and spans nearly 16 kb. Two variants of UGT2B7 have been described, with histidine or tyrosine at position 268.¹ Previous studies have indicated that this polymorphism minimally influences enzyme activity and substrate specificity of UGT2B7.^{2,9,10} However, large inter-individual variation in the ability to glucuronidate morphine^{11–13} and AZT¹⁴ indicates that other polymorphisms may be present in UGT2B7. To investigate this possibility, we have screened the coding and regulatory regions of the *UGT2B7* gene for genetic variation. Moreover, the current study addresses the impact of *UGT2B7* gene variants on the catalysis of morphine glucuronidation in cancer patients.

RESULTS

Analysis of *UGT2B7* gene sequence variation

All six exons and the 5' regulatory sequences of the human *UGT2B7* gene, covering the coding regions and exon–intron boundaries important for mRNA splicing, were amplified from genomic DNA and sequenced. A schematic presentation of the gene structure and PCR amplification strategy is shown in Figure 1. We were unable to obtain reliable sequence readings of the 3' untranslated region of exon 6, probably because of polymerase slippage in an adenine-rich region of the sequence. For an initial screen, we identified 17 individuals with low morphine glucuronide to morphine serum ratios (M6G/morphine: 1.4 ± 0.5 ; M3G/morphine: 13.8 ± 15.3), and sequence analyses were carried out for all indicated gene fragments of these patients. This strategy

increases the probability of detecting functional UGT2B7 polymorphisms. The screen was extended for selected *UGT2B7* gene fragments (immediate 5' region, exons 2 and 4) to include a total of 52 individuals (29 individuals with low morphine glucuronide to morphine serum ratios; M6G/morphine, 1.9 ± 0.6 and M3G/morphine, 14.0 ± 11.3 ; and 23 individuals with high morphine glucuronide to morphine serum ratios: M6G/morphine, 12.2 ± 4.1 and M3G/morphine, 82.2 ± 33.0).

In the 5' regulatory region, 13 deviations from the reference sequence (GenBank entry AF282881) were found. Seven of these deviations occurred on both gene copies in all individuals examined, suggesting that these sequencing discrepancies are sequencing artifacts in the reference sequence or, less likely, rare haplotypes not observed in our sample. These deviations included a T instead of C at position 154 (154C>T), insertions of G after positions 200 (200–201insG), 216 (216–217insG) and 277 (277–278insG), deletion of A at position 964 (964delA), and insertion of A after positions 1078 (1078–1079insA) and 1090 (1090–1091insA) (positions given according to GenBank entry AF282881). For clarity, an updated version of the 5' sequence is presented in Figure 2, and all further nucleotide positioning in the 5' region is given according to the numbering in this figure.

A total of 12 nucleotide variations, six in the 5' regulatory region and six within the exonic sequences, were found, all of which were single nucleotide polymorphisms (SNPs). No polymorphisms were found in exons 3, 5 and 6. One SNP in exon 1 was observed in one individual only. Whereas all nucleotide substitutions in the 5' sequences were transition mutations, two of the substitutions within the coding sequences were transversions. The type and position of the SNPs, and their frequency of occurrence, are listed in Table 1. Only one of the identified SNPs (pos. 816 in exon 2) gives rise to amino acid substitution (His268Tyr). None of the SNPs were located close to exon–intron boundaries or in sequences believed to be important for splice site recognition.

UGT2B7 Genotypes and Haplotypes

Seven *UGT2B7* genotypes were observed among 239 individuals who were genotyped by DNA sequencing and/or hybridization probe analysis. For clarity, sequences identical to the sequences in Figure 2 and NM_001074 are referred to as wild type. We consistently observed linkage between the

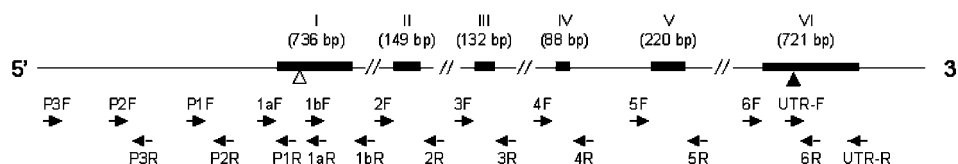


Figure 1 PCR amplification and sequencing screen of the human *UGT2B7* gene. The complete coding region and 1.4 kb of the 5' flanking sequences were amplified by PCR and sequenced. Exons are indicated by black boxes and numbered with roman numbers. The sizes of exons are indicated. Start and stop codons are indicated by open and filled triangles, respectively. Forward (F) and reverse (R) PCR and sequencing primer pairs are indicated by horizontal arrows. The sizes of introns range from 0.7 kb (intron 4) to 4.2 kb (intron 5).

5' -ATAGTCCAATTACACTGAGACGTCAGATTT
-1300 GGCAGCAAAGAAGGAGTTTAAATAATTGCAGGGCACTGAGCAAGGAGATAA
-1250 GAAG**A**GAACCC**T**AAATCCAGCTCCTCAAGGAGTTCTGGGTTGGAGGTTT
-1200 AAGGAGATCATAGATGGTGATAGGCTGGAAAAATTGGGTAATTGATTAGT
-1150 CAGGGTAATAGGGTAAAGTCATCAGAACAAGAACTACATTTTGGCTGA
-1100 GTCAGTTCCCTTGGGGTCTTCTAGATCAGCTGGCATCAGTGAAGTCT**TT**
-1050 CAGATCAGCTGCATCAGTGCAGATCCCTCAGACCAGCCGAGTCAGTAGTT
-1000 TTAGCAGTATGCTGGACCTGAAGGAATACCTCAAAGGAAAGTTGATCAT
-950 TTCATAATGTTTAAAGTTAATATTTATAGAAGGATTAAGAGGAACATAGT
-900 CTTGTAACAGGGTCTGCATAAATCTAGGACAACAGCCAAATAACTGTGAG
-850 GAAGTGAGT**CG**GAGAACAAAGCTAACCTAAATGATTAATGCTGAATGACTA
-800 CAAGCTTGGTTTACTTTTCTCTTTTCTTTCTTTTATCTTTGATTACTTTA
-750 ACAAGTTTAAAAAGATAGTGTCTATCTTGAACCTCTCAATATCTTTACATG
-700 ATGGTTGTAAGAATCAACATGTATACGCTATATCATAAATGAACCTTTAA
-650 AATATTAATGTACATAAAAAGAAGCCAGTCACAAAATACCACATATTGTAT
-600 GATCTATGCATTAATAAATGTCCAGAAATAGGCCAAATCTATAGAGATAGA
-550 AATCAATTAGCAATACCTAGGACAGGGGAAATGCAGAACTAGGGGTG
-500 GAGAGAAAAGGCTAAGGACTATAGGGCTTATTTTGGGGAGATAAAAAGGG
-450 CTCTCCAAGTATGTTTATGGTAGATGCACAATCTGTGAATATACTATG
-400 AAACATTTAAATTTACACAATAAATGATAAATAGTATGATATTTAAATTA
-350 CATGTCAAACAAGTTTACAAAAATATGTGGACCATGTTTAGTCATTTAA
-300 TCTTTAGTTTGTGTCATGGACTGCAGAAAACA**A**GATCTGTCACTGCTACT
-250 GTTCTGGACACTCTTCTAAAATATATGTCATAAGACAGATGGCATGTCCA
-200 TACAAGATCCTTGATATTAGCTGAAGGATAGCACTCATAAACATAAAAAGG
-150 GAAATTAATCACATCTGTGTGAACAGATCATTACCTTCATTTGCTCT**TT**
-100 TGCCATCCACATGCTCAGACTGTTGATTAAATGATATGATATGACTTTG
-50 ACTTATAAGGGTTACATTTTAACTTCTTGGCTAATTTATCTTTGGACATA
+1 **A**CCATGAGAAATGACAGAAAGGAACAGCAACTGGAACAAGCATTGCAT
50 TGCACCAGG**ATG**TCTGTGAAATGGACTTCAGTAATTTTGCATAATACAAC
100 GAGCTTTTGGCTTAGCTCTGGGAATGTGGAAAGGTGCTGCTGGGCAG
150 CAGAATACAGCCATTGGATGAATATAAAGACAATCTCGGATGAGCTT-3'

Figure 2 Nucleotide sequence of the *UGT2B7* gene proximal promoter. Positions showing nucleotide variation are in bold and are underlined. The translation start codon is boxed. The major transcription start site (+1, in bold) is according to Ishii et al.²²

six SNPs in the 5' regulatory region in 17 individuals analyzed by DNA sequencing (see Table 1). Therefore, hybridization probe analysis at one variant position (-102 T/C) was used to predict the complete set of 5' SNPs in all other subjects. A total of 41 individuals were homozygous variant for the 5' SNPs, but consistently homozygous wild-type TC at positions 815-816 in exon 2, and 25 of these individuals were also homozygous for the variants A at position 749 (exon 2) and C at position 1076 (exon 4). No individuals carried either 749 A or 1076 C alone. In addition, all individuals homozygous for the variant AT at positions 815-816 were also homozygous for the variant C at positions 1073 and 1076.

As a result of the consistent linkage between SNPs, a reliable prediction of the specific combination of variants, haplotypes, on each of the two chromosomes of each individual could be performed. The three main haplotypes predicted are illustrated in Figure 3. A fourth haplotype contained all variants of haplotype B and in addition an A to G transition at nucleotide position 386 in exon 1 (Arg124Arg). This haplotype was observed for 1/34 chromosomes, but was not characterized further. No individuals were homozygous variant at all variable positions. A comparison of the observed and predicted (Hardy-Weinberg equilibrium) genotype frequencies regarding different *UGT2B7* polymorphisms showed that the observed genotype distribution was in agreement with the predicted distribution.

Relationship between *UGT2B7* Genotypes and Morphine Glucuronide to Morphine Serum Ratios

To analyze whether *UGT2B7* polymorphisms correlate with morphine glucuronide to morphine serum ratios, we determined the *UGT2B7* genotype and haplotype distributions in 175 of the 239 cancer patients. These patients had normal renal and hepatic function and received morphine

Table 1 *UGT2B7* gene polymorphisms

Position	Number of chromosomes analyzed	Wild-type seq.	Polymorphism	Affected codon	Observed frequency of occurrence (variant)	Estimated frequency* of occurrence (variant)
-1246, promoter	34	A	A/G		0.35	0.44
-1239, promoter	34	T	T/C		0.35	0.44
-1052, promoter	34	T	T/C		0.35	0.44
-840, promoter	34	G	G/A		0.35	0.44
-268, promoter	104	A	A/G		0.39	0.44
-102, promoter	478	T	T/C		0.44	0.44
386, exon 1	34	A	agA/agG	Arg124Arg	0.03	<0.03
749, exon 2	478	G	acG/acA	Thr145Thr	0.89	0.89
815, exon 2	478	T	ccT/ccA	Pro267Pro	0.56	0.56
816, exon 2	478	C	Cat/Tat	His268Tyr	0.56	0.56
1073, exon 4	104	G	ctG/ctC	Leu343Leu	0.61	0.56
1076, exon 4	104	T	taT/taC	Tyr344Tyr	0.86	0.89

The positions indicated for promoter variants refer to the numbering in Figure 2. Positions indicated for exonic variants refer to the numbering of GenBank entry NM.001074. The positions of variation within codons are indicated by uppercase letters.

*Estimated frequencies are based on the observation of consistent linkage between sets of SNPs from 478 chromosomes.

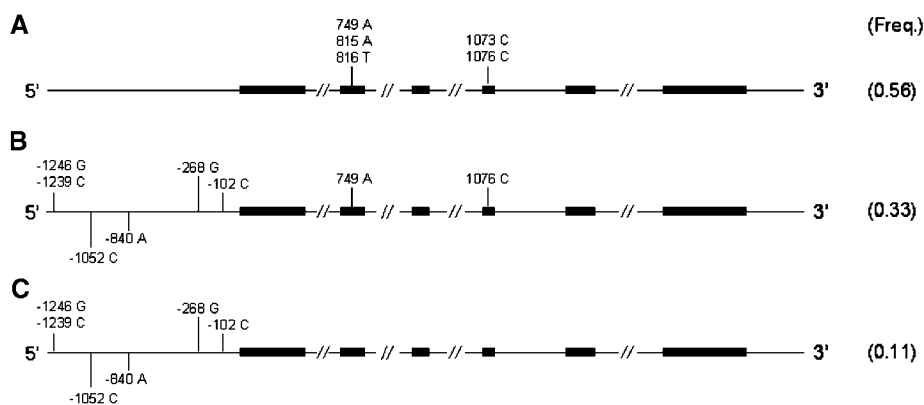


Figure 3 *UGT2B7* haplotypes. Haplotypes were predicted from genotyping of 239 subjects by DNA sequencing and/or hybridization probe analysis. Shown are the three main haplotypes. A fourth haplotype was observed in 1/34 chromosomes and contained the SNPs of haplotype B and an A to G transition at nucleotide position 386 in exon 1.

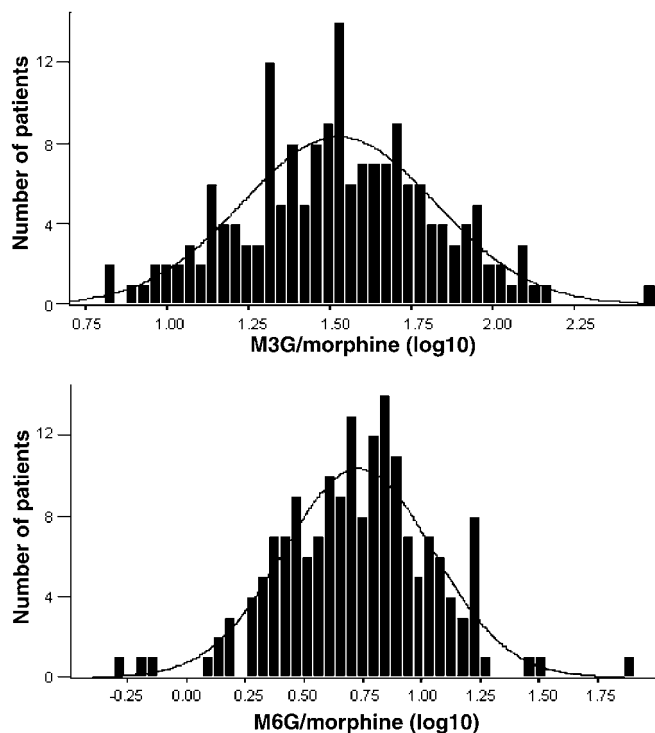


Figure 4 Histogrammic distribution of morphine glucuronide to morphine serum ratios among 175 cancer patients on chronic oral morphine therapy and who had normal hepatic and renal function.

by the oral route. The M3G/morphine, M6G/morphine and M3G/M6G serum ratios varied 145-, 48- and 7-fold, respectively, among individuals. The distributions of the M3G/morphine and M6G/morphine serum ratios for the 175 patients are shown in Figure 4, and the mean serum ratios calculated for each *UGT2B7* genotype are shown in Table 2. The histograms provided no evidence for bi- or multimodal distribution of glucuronide to morphine serum

ratios. No statistically significant intergenotypic differences were observed ($P > 0.05$; one-way ANOVA).

We also compared the distribution of *UGT2B7* genotypes and haplotypes among the 30 individuals who had the lowest morphine glucuronide to morphine serum ratios with the corresponding distribution among the 30 individuals having the highest serum ratios. As shown by the data presented in Tables 3 and 4, *UGT2B7* genotypes and haplotypes were evenly distributed between individuals with low and high ratios.

***UGT2B7* major promoter activity assayed by reporter gene analysis**

The regulatory region of the *UGT2B7* promoter consists of two domains, each with features of independent promoters.⁸ Primer extension analysis has revealed that the region from -1 to -400 most probably contains the major promoter.^{8,15} To investigate the potential significance of the alternative nucleotides at positions -268 and -102 of the major promoter for *UGT2B7* gene expression, the plasmids pGL3-2B7wt and pGL3-2B7mut, containing the wild-type and variant promoter sequences, respectively, were transiently transfected into HepG2 cells and tested for the ability to drive the firefly luciferase reporter gene. Both constructs were active in HepG2 cells. As shown in Figure 5, slightly higher luciferase activity was observed with the pGL3-2B7mut construct than with the pGL3-2B7wt construct, but the differences failed to reach statistical significance ($P = 0.155$; one-way ANOVA).

DISCUSSION

We have identified polymorphisms in the coding and regulatory regions of the human *UGT2B7* gene and described their distribution in a Caucasian population consisting of 239 cancer patients. Among the 12 different polymorphisms identified, six were localized in the regulatory region and six were within exons. Only one SNP in exon 2 changes the amino acid sequence. The resulting variants, *UGT2B7* 268H and *UGT2B7* 268Y, have been

Table 2 *UGT2B7* genotypes and glucuronide/morphine serum ratios (mean \pm SD; range)

Genotype	M6G/morphine	M3G/morphine	M3G/M6G
A/A (n=55)	7.2 \pm 9.8 (0.5–72.8)	41.4 \pm 44.1 (6.4–309.2)	6.7 \pm 2.9 (3.7–16.4)
A/B (n=64)	7.1 \pm 5.6 (0.7–30.8)	44.0 \pm 28.0 (7.7–126.3)	6.9 \pm 3.1 (3.1–20.7)
A/C (n=26)	7.0 \pm 4.7 (2.3–16.7)	44.8 \pm 32.6 (13.0–142.0)	6.4 \pm 1.3 (4.1–8.9)
B/B (n=16)	6.5 \pm 3.1 (1.5–12.5)	38.5 \pm 21.3 (11.8–82.5)	6.0 \pm 1.3 (3.8–8.3)
B/C (n=12)	7.2 \pm 5.2 (1.4–16.0)	44.3 \pm 34.6 (9.3–129.0)	6.3 \pm 1.5 (4.6–9.1)
C/C (n=2)	2.4 \pm 0.8 (1.9–3.0)	17.5 \pm 10.8 (9.9–25.2)	6.8 \pm 2.2 (5.3–8.4)

Table 3 Distribution of *UGT2B7* genotypes among individuals with low and high glucuronide to morphine, and M3G to M6G serum ratios

Genotype	M6G/morphine (range)		M3G/morphine (range)		M3G/M6G (range)	
	(0.5–2.7)	(11.0–72.8)	(6.4–17.9)	(64–309.2)	(3.1–4.6)	(8.3–20.7)
A/A	11	8	11	9	13	10
A/B	10	11	10	11	11	14
A/C	3	6	5	5	2	4
B/B	2	2	1	2	3	0
B/C	3	3	2	3	1	1
C/C	1	0	1	0	0	1

Each group consisted of the 30 individuals having the lowest or highest serum ratios among 175 patients receiving oral morphine. Absolute numbers are shown.

Table 4 Relative distribution of *UGT2B7* haplotypes among individuals with low and high glucuronide to morphine and M3G to M6G serum ratios

Haplotype	M6G/morphine (range)		M3G/morphine (range)		M3G/M6G (range)	
	(0.5–2.7)	(11.0–72.8)	(6.4–17.9)	(64–309.2)	(3.1–4.6)	(8.3–20.7)
A	0.58	0.55	0.62	0.57	0.65	0.63
B	0.28	0.30	0.23	0.30	0.30	0.25
C	0.14	0.15	0.15	0.13	0.05	0.12

Each group consisted of the 30 individuals having the lowest or highest serum ratios among 175 patients receiving oral morphine.

extensively studied both *in vitro* and *in vivo*, and basically no significant differences in substrate specificity or catalytic activity have been detected.^{2,9} Although we found that the H268Y polymorphism cosegregates with four additional SNPs in exons 2 and 4, the silent nature of these SNPs would suggest no effects of those polymorphisms, which is consistent with previous findings⁹ and the results of the present study. Except for a rare SNP in exon 1, which was observed in one heterozygous individual only, all variants exhibited a frequency of 11% or more in the population. Based on the observation of consistent linkage between SNPs, three main haplotypes could be predicted, two of which were found to contain all six novel nucleotide variations in the 5' regulatory region (haplotypes B and C). The tight linkage of SNPs correlates well with the relatively small size of the *UGT2B7* gene (about 16 kb) and the low probability of recombinational events within short distances along the chromosome.

UGT2B7 is the only enzyme demonstrated to promote the glucuronidation of both the 3- and 6-hydroxyl moieties of morphine.³ Consequently, variation within the *UGT2B7* gene has been considered a reasonable explanation for the variability in morphine glucuronidation in humans. However, although we observed a considerable interindividual variation in glucuronide to morphine serum ratios, the distribution of the ratios approximated a normal distribution and in this regard differs from what is observed for some of the most important cytochrome P450 (CYP) enzymes taking part in drug metabolism in humans, such as CYP2D6, where poor (PM), extensive (EM) and ultrarapid metabolizer (UM) phenotypes can be predicted from the trimodal distribution of debrisoquine and codeine oxidation caused by CYP2D6 gene mutation (PM) or gene duplication (UM).¹⁶ The near unimodal distribution of morphine glucuronide to morphine serum ratios suggests that defective alleles of *UGT2B7* or functionally active duplications are unlikely to occur in the population at clinically relevant

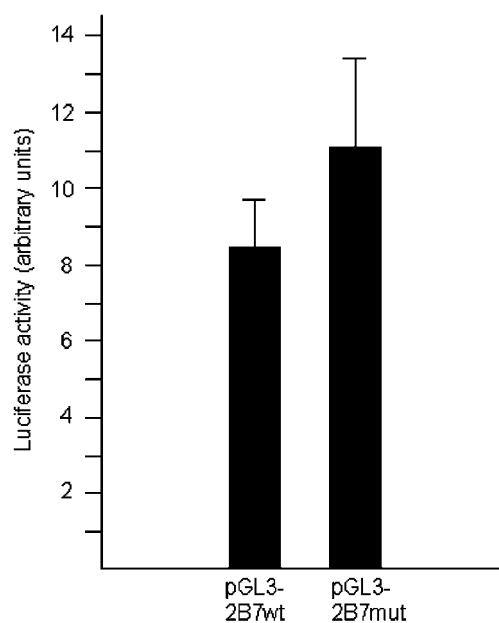


Figure 5 Relative luciferase activity of transfected *UGT2B7* promoter constructs. Relative firefly luciferase activities were calculated as the mean of triplicate assays performed with each of four (pGL3-2B7wt) and five (pGL3-2B7mut) independent plasmid clones and normalized to *Renilla* luciferase activities.

frequencies, and is consistent with our data implying that variation in morphine glucuronide to morphine ratios may not result from structural variation in *UGT2B7*. The apparent absence of functional protein polymorphisms suggests that the *UGT2B7* gene is under high selective pressure, and probably reflects its important role in the biotransformation of both endogenous and xenobiotic substrates.

Multiple forces may act on base composition in the coding regions of mammalian genes. Variation may be less favorable if it occurs in sequences important for splice-site selection or if mRNA transport and translation are subject to DNA structural constraints, and for some amino acids tRNA abundance may affect protein synthesis. Although it has been well documented from bacterial systems that the use of synonymous codons may affect the expression levels of genes,^{17,18} it is still a matter of debate whether this is also the case in human cells.^{19–21} Our results, showing no intergenotypic differences in morphine glucuronidation, indicate that overall *UGT2B7* expression is not significantly affected by the codon variations described here. It is, however, important to recognize that morphine glucuronide to morphine serum ratios may also be affected by variation in other metabolic processes such as cross-membrane transport, hepatic blood flow, renal elimination and volume of distribution, which could make minor differences in *UGT2B7* expression functionally undetectable. One should also have in mind that patients with malignant disease are not an easy group to study because of huge variation in daily morphine dosage, medical conditions and additional

medications, all representative of the typical problems in achieving adequate pain control, but which may contribute to obscuring a possible genotype–phenotype relationship.

The *UGT2B7* gene promoter displays a complex structure with two domains, P1 and P2, which harbors the features of two independent promoters.⁸ The major transcription initiation site for hepatic and intestinal mRNAs was mapped to the P1 domain, which may be the major promoter. In a study by Ishii *et al*,²² three main regions denoted A, B and C were identified within the P1 domain that were resistant to DNase I digestion in nuclear extracts from HepG2 cells, suggesting that these regions may bind factors important for *UGT2B7* transcription in human liver. Interestingly, the –102 T/C polymorphism is located within the C region (–114 to –89), but the factor(s) that bind to this region in HepG2 cells remains to be determined. Transient transfection experiments indicated that the variant P1 promoter containing –268 G and –102 C was slightly more active than the wild-type promoter in HepG2 cells, but whether these polymorphisms may contribute to differential expression of *UGT2B7* in the liver is uncertain. Our results with morphine glucuronidation did not reveal significant differences between individuals carrying variant and wild-type promoters, suggesting that these nucleotide variations do not contribute to the variability in morphine glucuronide to morphine serum ratios in cancer patients.

A high concentration of putative binding sites for the intestinal transcription factor CDX2 in the P2 promoter indicates that this region may be important for expression in the gastrointestinal (GI) tract.⁸ The GI tract is the first large metabolic organ encountered by orally ingested compounds and represents a significant localization for extrahepatic metabolism. Interestingly, whereas little variation has been reported for hepatic *UGT2B7* expression, the expression of intestinal *UGT2B7* mRNA and protein shows considerable variation among individuals.²³ The reason for this variation is not known, but such variation could undoubtedly have a significant impact on the therapeutic efficacy or toxicity of drugs because of variation in intestinal glucuronidation during or prior to absorption. A computer search with wild-type and variant promoter sequences using MatInspector professional, release 5.2, against the vertebrate group from the Matrix Family Library Version 2.3 database (http://www.genomatix.de/mat_fam) indicated that the –1246 A/G and –1239 T/C polymorphisms generate new putative sites for the binding of gut-enriched Kruppel-like factor, NF-kappaB, and Ras-responsive element binding factor in the variant sequence, whereas a putative Oct-6 binding element is lost. Whether these differences in the promoter region may contribute to polymorphic intestinal *UGT2B7* expression remains to be investigated. It is also interesting to note that two putative progesterone response elements are present on the positive strand (–1133 to –1107 and –844 to –818), one of which covers the +840 G/A polymorphism. *UGT2B7* has high activity toward progesterone,²⁴ and these elements could possibly play a role in the regulation of *UGT2B7* in steroid hormone metabolism. It is

not clear whether the –840 polymorphism has implications for transcription factor binding.

In summary, we have demonstrated the presence of several single nucleotide polymorphisms in the coding and regulatory regions of human *UGT2B7* gene, which give rise to four different haplotypes and seven genotypes. The most surprising observation was, except for the previously described H268Y polymorphism, the complete absence of structural variation in *UGT2B7*. Our strategy, including in the genetic screen a significant number of individuals with very low morphine glucuronide to morphine serum ratios, should increase the likelihood of detecting functional polymorphisms if they were present in the population at clinically relevant frequencies. This implies that factors other than *UGT2B7* polymorphism may be more deciding for the variability in morphine metabolite to morphine serum ratios. Interestingly, Toide *et al*²⁵ recently presented evidence suggesting that hepatocyte nuclear factor-1 alpha (HNF-1 α) may be a limiting factor in hepatic *UGT2B7* mRNA expression. Therefore, interindividual variation in HNF-1 α expression may contribute to variability in morphine metabolite to morphine serum ratios. In addition, given the diversity of glucuronidation enzymes in the GI tract and the recent demonstration of polymorphic expression in the intestine of several UGT isoforms including *UGT2B7*, the contribution of prehepatic metabolism to the variability in morphine glucuronidation may be significantly underestimated. The data presented here provide convincing evidence that functional and structural variations in *UGT2B7* may not be frequently found in the Caucasian population. This new information may contribute to increased understanding or valuable reconsideration of the biochemical basis of variation in metabolism of not only morphine, but also a variety of compounds conjugated by *UGT2B7*.

MATERIALS AND METHODS

The study protocol was approved by the Regional Committee for Research Ethics, Health Region IV, Norway. Written informed consent was obtained from each subject.

Patient Demographics and Morphine Administration

A total of 239 patients with malignant disease using morphine to relieve pain were included in the study and genotyped for polymorphisms in the *UGT2B7* gene. All patients were admitted to St Olav's University Hospital in Trondheim, a 900-bed tertiary hospital. Patients below 18 years of age, those not consenting to participate in the study, or those not competent in the Norwegian language were not included. The start of scheduled morphine treatment was at least 3 days prior to inclusion.

A subgroup consisting of 175 patients with normal renal and hepatic function and who received slow-release morphine by the oral route were included in the study of correlation between *UGT2B7* genotype and morphine glucuronide to morphine serum ratios. Of these patients, 48 received additional immediate-release morphine for breakthrough pain. Rescue morphine was administered at least 6 h prior to blood sampling. Patient demographics for

Table 5 Demographics of 175 patients included in the study of correlation between *UGT2B7* genotype and morphine glucuronide to morphine serum ratios

Number of patients	175
Gender male:female	97:78
Age in years (mean (range))	62 (29–88)
Tumor diagnosis:	
Prostate	33
Other urological	10
Lung	37
Breast	40
Gastrointestinal	21
Hematological	13
Others	10
Unknown origin	11
GFR (ml/min/1.73 m ²); SD	88 ± 19
Albumin (g/l); SD	33 ± 5
ALAT activity (U/l); SD	35 ± 42
Time in months since start of morphine (mean (range))	4 (0.2–52)

the 175 patients are listed in Table 5. Aspartate aminotransferase activity (ASAT) <500 U/l and/or alanine aminotransferase activity (ALAT) <150 U/l were considered acceptable since moderate hepatic impairment is not associated with changes in morphine metabolism or clearance.^{11,26} Patients having estimated glomerular filtration rate (GFR) <60 ml/min/1.73 m² ($GFR = 186.3 \times (\text{serum creatinine in mg/dl})^{-1.154} \times \text{age (years)}^{-0.203} (\times 0.742 \text{ if patient is female})$)²⁷ were not included. Gastrointestinal pathology was not considered a criterion for exclusion since potential differences in absorption (in analogy with different dose) is not believed to significantly influence the serum metabolite to morphine ratio.

All patients received additional medications. The most frequently given drugs were corticosteroids, acetaminophen, antiemetics, benzodiazepines, antibiotics and non-steroidal anti-inflammatory drugs. A complete list of comedications is available on request.

Blood Sampling and Serum Analyses

Venous blood samples were collected in the morning, in general 1–2 h after morphine dosing. Within this time interval, the M3G/morphine and M6G/morphine serum ratios show little variation in patients receiving slow-release morphine.¹² Serum was separated from whole blood by centrifugation at 3000 g for 10 min, and stored at –85°C prior to analysis of morphine, M3G and M6G. Serum morphine, M3G and M6G concentrations were quantified at the Department of Clinical Pharmacology at the University Hospital in Trondheim, using liquid chromatography mass spectrometry (LC/MS) as described.²⁸ The limits of detection were 0.35 nmol/l for morphine and 2.2 nmol/l for M3G and M6G. The analytical coefficients of variation were: morphine 3.0%; M6G 5.5% and M3G 7.0%.

Serum concentrations of creatinine, ASAT and ALAT were determined by standard methods at the Department of

Clinical Chemistry, St Olav's University Hospital, Trondheim.

DNA Isolation

Genomic DNA was prepared from 50–200 µl EDTA blood on a MagNA Pure LC (Roche) using the MagNA Pure LC DNA Isolation Kit I with the high-performance protocol following the instructions of the corresponding package inserts. Purified genomic DNA was eluted in 100 µl elution buffer and stored at –20°C.

Oligonucleotide Design and PCR Conditions

PCR primers were designed to allow amplification of each of the six exons, intron–exon boundaries, and the 5' regulatory region of the human *UGT2B7* gene. PCR amplifications were performed in 20 µl reactions on a LightCycler System (Roche) using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche), 1–2 µl of purified genomic DNA, primers at 0.5 µM and 4 mM Mg²⁺, unless stated otherwise. All amplifications included a 7 min initial denaturation step at 95°C. The regions amplified, sequences of the PCR primers, and the cycling conditions are given in Table 6. Amplification of the 3' UTR using different sets of primer pairs did not result in reliable sequence readings (conditions not shown).

DNA Sequencing

PCR products were purified using the CONCERT™ Rapid PCR Purification System (GibcoBRL) and eluted in 50 µl nuclease-free H₂O. DNA cycle sequencing was carried out in 20 µl reactions on a Perkin-Elmer GenAmp PCR system 9600 (Perkin-Elmer, Foster City, CA, USA) using 6 µl of purified

PCR product, 3.2 pmol primer, and ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA) as described by the manufacturer. The forward and reverse sequencing primers were the same as those used in the respective PCR amplifications. Conditions for the sequencing of PCR products were 25 cycles with 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Residual dideoxy terminators were removed by ethanol precipitation and sequences analyzed on an Applied Biosystems 377 DNA sequencer.

Hybridization Probe Genotyping

The T/C polymorphism at position –102 in the promoter, and the 749 G/A and 815.816 TC/AT polymorphisms in exon 2 were detected using the LightCycler (Roche Diagnostics) fluorescence resonance energy transfer method.²⁹ The sequences of the fluorescence labeled probes (TIB MOLBIOL, Berlin, Germany) are given in Table 7. For all hybridization probe pairs, the anchor and sensor probes were spaced by 1 bp, and all unlabeled 3'-ends were phosphorylated to block extension of the probe. PCR conditions were as described above for the relevant gene fragments, except that the time of annealing was increased from 5 to 10 s and the extension time for the –102 promoter polymorphism was increased from 22 to 24 s. Hybridization probes were used at 0.2 µM concentration. After PCR amplification, the polymorphisms were detected by melting curve analysis using the following segment parameters: for the –102 T/C polymorphism, 95°C for 0 s, 68°C for 30 s, 60°C for 30 s, 55°C for 30 s, 40°C for 0 s, followed by a

Table 6 *UGT2B7* PCR primers and amplification conditions

Amplified region	Fragment size (bp)	PCR primers (forward/reverse)	PCR cycling conditions			
			Denat.	Annealing	Extension	Cycles (n)
5' region, 34–523 (GenBank entry AF282881)	490	5'-ATAGTCCAATTACACTGAGACG-3'/ 5'-TCCGACTCACTTCCTCACAG-3'	95°C, 15 s	64°C, 5 s	72°C, 20 s	40
5' region, 474–934 (GenBank entry AF282881)	461	5'-CTGCATAATTCTAGGACAAC-3'/ 5'-CTACCATAACAATCAGTTGG-3'	95°C, 15 s	57°C, 5 s	72°C, 19 s	40
5' region, 896–1469 (GenBank entry AF282881)	574	5'-GGGGAGATAAAAGGGCTCTC-3'/ 5'-CAAAAGCTCAGTTGTATTAGCAA-3'	95°C, 15 s	62°C, 5 s	72°C, 22 s	50
Exon 1 (82 bp upstream ATG, 369 bp of the CDS)	451	5'-TGGCTAATTTATCTTTGGAC-3'/ 5'-AGTTATGTCACCAATATTG-3'	95°C, 15 s	50°C, 5 s	72°C, 20 s	50
383 bp of exon 1, 26 bp of intron 1	409	5'-GGAAATCATGTCAATTTGG-3'/ 5'-GTTACTGAGTGAAAAAATAC-3'	95°C, 15 s	55°C, 5 s	72°C, 25 s	40
26 bp of intron 1, exon 2, 29 bp of intron 2	204	5'-ACCTTTTTTTTTCTATTCCTGT-3'/ 5'-CAAAATAAAACCAACAAAAGTATG-3'	95°C, 15 s	58°C, 5 s	72°C, 8 s	40
24 bp of intron 2, exon 3, 27 bp of intron 3	183	5'-ATAAAGCAAATCTTTCTTCAC-3'/ 5'-CCACACCAGTAAGGCGCTTC-3'	95°C, 15 s	60°C, 5 s	72°C, 8 s	10
24 bp of intron 3, exon 4, 24 bp of intron 4	136	5'-AAAATATTTTCTTTATTGTAACAG-3'/ 5'-AGTATTTGTTCCACAGATC-3'	95°C, 15 s	56°C, 5 s	72°C, 6 s	40
9 bp of intron 4, exon 5, 2 bp of intron 5	231	5'-CAATCCTAGGTCATCCAAAG-3'/ 5'-ACGAAGGATCATTAACTACTC-3'	95°C, 15 s	60°C, 5 s	72°C, 8 s	5
24 bp of intron 5, 337 bp of exon 6 (comprising the 280 bp CDS)*	361	5'-ACTGTCTTTATTTTATCTTC-3'/ 5'-GAATAAACTGAAGTAGTCTC-3'	95°C, 15 s	55°C, 5 s	72°C, 15 s	10
			95°C, 15 s	53°C, 5 s	72°C, 15 s	40

*3 mM Mg²⁺ was used in the PCR.

Table 7 Hybridization probes used for genotyping

Name	Probe sequence	Nucleotide detected
ProAnchor	5' LC Red640-CCACATGCTCAGACTGTTGATTTAATGATA-3'/	
Pro[C]sensor	5'-ACCTTCATTTGTCTCCTTGCCA-FI-3'	-102 C
749Anchor	5'-CCATACGTCAGCTTTCCCCATTGTC-FI-3'/	
749Sensor	5' LC Red705-CAGATAACGTAGTGGGTCTTCCTGA-3'	749 C
815.816Anchor	5' LC Red640-CCAAATGTTGATTTTGTGGAGGACTCC-3'/	
815.816Sensor	5'-GAATTTTCAGTTCCATATCCACTCTT-FI-3'	815 A+816 T

LC Red640: LightCycler Red640; LC Red705: LightCycler Red705; FI: fluorescein.

temperature increase to 73°C at 0.1°C/s; and for simultaneous detection of the 749 G/A and 815.816 TC/AT polymorphisms, 95°C for 15 s, 66°C for 30 s, 61°C for 30 s, 55°C for 30 s, 40°C for 30 s, followed by a temperature increase to 85°C at 0.1°C/s.

UGT2B7 Promoter Constructs

Reporter constructs containing wild-type and variant (G at position -268 and C at position -102) sequences of the *UGT2B7* major promoter were generated from genomic DNA by PCR and insertion of the PCR products into the pGL3-basic vector (Promega, Madison, WA, USA). The following oligonucleotide primers with *Xho*I or *Hind*III sites (underlined) were used to amplify the region from -466 to +12 (according to Figure 2) of the *UGT2B7* promoter: 5'-CCGCTCGAGTGGGGAGATAAAAGGGCTCTC-3' (forward) and 5'-TCCCAAGCTTATTTCTCATGGTTATGTCCAAAG-3' (reverse). The PCR was performed with AmpliTaq polymerase (Perkin-Elmer) using 2.5 mM dNTPs and 3 mM Mg²⁺ under the following cycling conditions: 95°C for 15 s, 60°C for 5 s, and 72°C for 22 s for 50 cycles. The resulting PCR products from homozygous wild-type and homozygous variant individuals were digested with *Xho*I and *Hind*III and subcloned into pGL3-basic vector at compatible sites to generate the plasmids pGL3-2B7wt and pGL3-2B7mut. DNA sequencing was carried out on both constructs to ensure that no undesired mutations had been introduced during DNA amplification by *Taq* polymerase.

Cell Culture and Transient Transfection

HepG2 cells were obtained from American Type Culture Collection (ATCC) and cultured in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum, L-glutamine (80 mg/l), sodium pyruvate (1 mM), fungizone (1 µg/ml) and gentamicin (50 µg/ml), and maintained at 37°C in 5% CO₂ and 95% air. Growth medium and additives were from Gibco. Transient transfections with pGL3-2B7wt and pGL3-2B7mut plasmids were performed using the nonliposomal formulation FuGENE 6 transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, 2 × 10⁵ HepG2 cells were seeded in 24-well culture dishes in 0.5 ml medium and grown overnight before transfection with 150 ng of the luciferase reporter plasmid and 30 ng of the internal control plasmid pRL-TK (Promega, Madison, WA, USA), using 3 µl FuGENE 6 for each microgram of DNA. Cell extracts were prepared 48 h post-

transfection, using passive lysis buffer (100 µl; Promega). The dual luciferase reporter assays were carried out using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). All transfections were carried out in triplicate and repeated at least twice.

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REFERENCES

- Jin C, Miners JO, Lillywhite KJ, Mackenzie PI. Complementary deoxyribonucleic acid cloning and expression of a human liver uridine diphosphate-glucuronosyltransferase glucuronidating carboxylic acid-containing drugs. *J Pharmacol Exp Ther* 1993a; **264**: 475-479.
- Coffman BL, King CD, Rios GR, Tephly TR. The glucuronidation of opioids, other xenobiotics, and androgens by human UGT2B7Y (268) and UGT2B7H (268). *Drug Metab Dispos* 1998; **26**: 73-77.
- Coffman BL, Rios GR, King CD, Tephly TR. Human UGT2B7 catalyzes morphine glucuronidation. *Drug Metab Dispos* 1997; **25**: 1-4.
- Barbier O, Turgeon D, Girard C, Green MD, Tephly TR, Hum DW, Belanger A. 3'-azido-3'-deoxythymidine (AZT) is glucuronidated by human UDP-glucuronosyltransferase 2B7 (UGT2B7). *Drug Metab Dispos* 2000; **28**: 497-502.
- Jin CJ, Miners JO, Burchell B, Mackenzie PI. The glucuronidation of hydroxylated metabolites of benzo[a]pyrene and 2-acetylaminofluorene by cDNA-expressed human UDP-glucuronosyltransferases. *Carcinogenesis* 1993b; **14**: 2637-2639.
- Vore M, Montgomery C, Meyers M. Steroid D-ring glucuronides: characterization of a new class of cholestatic agents. *Drug Metab Rev* 1983; **14**: 1005-1019.
- Worrall S, Dickinson RG. Rat serum albumin modified by diflunisal acyl glucuronide is immunogenic in rats. *Life Sci* 1995; **56**: 1921-1930.
- Radomska-Pandya A, Little JM, Czernik PJ. Human UDP-glucuronosyltransferase 2B7. *Curr Drug Metab* 2001; **2**: 283-298.
- Holthe M, Klepstad P, Zahlsen K, Borchgrevink PC, Hagen L, Dale O et al. Morphine glucuronide to morphine serum ratios are unaffected by the *UGT2B7* H268Y and *UGT1A1**28 polymorphisms in cancer patients on chronic morphine therapy. *Eur J Clin Pharmacol* 2002; **58**: 353-356.
- Bhasker CR, McKinnon W, Stone A, Lo AC, Kubota T, Ishizaki T, Miners JO. Genetic polymorphism of UDP-glucuronosyltransferase 2B7 (UGT2B7) at amino acid 268: ethnic diversity of alleles and potential clinical significance. *Pharmacogenetics* 2000; **10**: 679-685.
- McQuay HJ, Carroll D, Faura CC, Cavaghan DJ, Hand CW, Moore RA. Oral morphine in cancer pain: influences on morphine and metabolite concentration. *Clin Pharmacol Ther* 1990; **48**: 236-244.
- Klepstad P, Kaasa S, Borchgrevink PC. Start of oral morphine to cancer patients: effective serum morphine concentrations and contribution

- from morphine-6-glucuronide to the analgesia produced by morphine. *Eur J Clin Pharmacol* 2000; **55**: 713–719.
- 13 Faura CC, Collins SL, Moore RA, McQuay HJ. Systematic review of factors affecting the ratios of morphine and its major metabolites. *Pain* 1998; **74**: 43–53.
- 14 Mentre F, Escolano S, Diquet B, Golmard JL, Mallet A. Clinical pharmacokinetics of zidovudine: inter and intraindividual variability and relationship to long term efficacy and toxicity. *Eur J Clin Pharmacol* 1993; **45**: 397–407.
- 15 Ishii Y, Takami A, Tsuruda K, Kurogi A, Yamada H, Oguri K. Induction of two UDP-glucuronosyltransferase isoforms sensitive to phenobarbital that are involved in morphine glucuronidation: production of isoform-selective antipeptide antibodies toward UGT1.1r and UGT2B1. *Drug Metab Dispos* 1997; **25**: 163–167.
- 16 Bertilsson L, Dahl ML, Dalen P, Al-Shurbaji A. Molecular genetics of CYP2D6: clinical relevance with focus on psychotropic drugs. *Br J Clin Pharmacol* 2002; **53**: 111–122.
- 17 Ermolaeva MD. Synonymous codon usage in bacteria. *Curr Issues Mol Biol* 2001; **3**: 91–97.
- 18 Smith NG, Eyre-Walker AJ. Why are translationally sub-optimal synonymous codons used in *Escherichia coli*? *Mol Evol* 2001; **53**: 225–236.
- 19 Kim CH, Oh Y, Lee TH. Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells. *Gene* 1997; **199**: 293–301.
- 20 André S, Seed B, Eberle J, Schraut W, Bültmann A, Haas J. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol* 1998; **72**: 1497–1503.
- 21 Duret L, Mouchiroud D. Determinants of substitution rates in mammalian genes: expression pattern affects selection intensity but not mutation rate. *Mol Biol Evol* 2000; **17**: 68–74.
- 22 Ishii Y, Hansen AJ, Mackenzie PI. Octamer transcription factor-1 enhances hepatic nuclear factor-1 α -mediated activation of the human UDP glucuronosyltransferase 2B7 promoter. *Mol Pharmacol* 2000; **57**: 940–947.
- 23 Strassburg CP, Kneip S, Topp J, Obermeyer-Straub P, Barut A, Tukey RH et al. Polymorphic gene regulation and interindividual variation of UDP-glucuronosyltransferase activity in human small intestine. *J Biol Chem* 2000; **275**: 36 164–36 171.
- 24 Jin CJ, Mackenzie PI, Miners JO. The regio- and stereo-selectivity of C19 and C21 hydroxysteroid glucuronidation by UGT2B7 and UGT2B11. *Arch Biochem Biophys* 1997; **341**: 207–211.
- 25 Toide K, Takahashi Y, Yamazaki H, Terauchi Y, Fujii T, Parkinson A et al. Hepatocyte nuclear factor-1 α is a causal factor responsible for inter-individual differences in the expression of UDP-glucuronosyltransferase 2B7 mRNA in human livers. *Drug Metab Dispos* 2002; **30**: 613–615.
- 26 Patwardhan RV, Johnson RF, Hoyumpa Jr A, Sheehan JJ, Desmond PV, Wilkinson GR et al. Normal metabolism of morphine in cirrhosis. *Gastroenterology* 1981; **81**: 1006–1011.
- 27 Manjunath G, Sarnak MJ, Levey AS. Estimating the glomerular filtration rate. Dos and don'ts for assessing kidney function. *Postgrad Med* 2001; **110**: 55–62; quiz 11.
- 28 Bogusz MJ, Maier RD, Driessen S. Morphine, morphine-3-glucuronide, morphine-6-glucuronide, and monoacetylmorphine determined by means of atmospheric pressure chemical ionization–mass spectrometry–liquid chromatography in body fluids of heroin victims. *J Anal Toxicol* 1997; **21**: 346–355.
- 29 Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* 1997; **22**: 176–181.