SEQUENCE VARIABILITY AND CANDIDATE GENE ANALYSIS IN TWO CANCER PATIENTS WITH COMPLEX CLINICAL OUTCOMES DURING MORPHINE THERAPY

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ABSTRACT:

In this case report, we present genetic differences in two morphine-related gene sequences, UDP-glucuronosyltransferase 2B7 (UGT2B7) and μ opioid receptors (MOR1), in two cancer patients whose clinical responses to morphine were very different [i.e., sensitive (patient 1) and low responder (patient 2)]. In addition, allelic variants in the UGT2B7 gene were analyzed in 46 Japanese individuals. Amplified DNA fragments for the two genes of interest were screened using single strand conformation polymorphism and then sequenced. In the UGT2B7 gene, 12 single nucleotide polymorphisms (SNPs) were newly identified with an allelic frequency ranging from 0.022 to 0.978. Six SNPs in the promoter region (A-1302G, T-1295C, T-1111C, G-899A, A-327G, and T-125C) and two coding SNPs (UGT2B7*2 in exon 2 and C1059G in exon 4) appeared to be consistently linked. Remarkable differences in the nucleotide sequence of UGT2B7 were observed between the two patients; in contrast to patient 1 who had “reference” alleles at almost SNP positions, but a rare ATTGAT*2(ATA) haplotype as homozygosity, patient 2 was a homozygous carrier for the predominant GCCAGC*1(TCG) sequence. Serum morphine and two glucuronide concentrations in patient 2 suggest that the predominant GCCAGC*1G sequence was not associated with a “poor metabolizer” phenotype. In the MOR1 gene, patient 1 had no SNPs, whereas patient 2 was a heterozygous carrier for both the G-1784A and A118G alleles. The present study describes substantial differences in genotype patterns of two genes of interest between the two patients. The results necessitate larger trials to confirm these observations in larger case control studies.

Morphine is the most important and widely used opioid analgesic in clinical medicine; however, interindividual differences in its effectiveness and in its side effects are major limitations for individualized pain treatment. In addition to the interindividual variability, ethnic differences in morphine pharmacokinetics and pharmacodynamics were reported (Cepeda et al., 2001). Recent clinical studies indicate that the large interindividual and inter-racial variability in drug responses occurs as a result of molecular alterations at the level of drug-metabolizing enzymes, drug targets/receptors, and drug transport proteins. In this regard, at least two genes are of interest as candidates, which might lead to large interindividual variability in clinical outcomes during morphine therapy. UGT2B7 is the predominant enzyme responsible for the glucuronidation of morphine to form M6G and M3G in humans (Coffman et al., 1997). M6G has been shown to be a potent analgesic in clinical studies, and the analgesic properties of morphine are enhanced by the action of M6G (Christrup, 1997). M3G, in contrast, has been shown to counteract the analgesic activity of morphine and M6G (Smith et al., 1990; Christrup, 1997). Since the reactivity of UGT2B7 with morphine leads to the production of very important and clinically relevant metabolites with a wide interindividual variability (Coffman et al., 1998), mutations of the UGT2B7 gene are potentially of pharmacological, toxicological, and physiological significance. However, genetic polymorphisms of UGT2B7 have not been well documented.

Morphine analgesia is produced by activation of opioid receptors within the central nervous system at both spinal and supraspinal levels. Like morphine, M6G is relatively selective for μ opioid receptors (MOR1) in the brain, binding to these receptors with higher affinity than to κ or σ receptors (Pasternak et al., 1987). The substantial interindividual differences in the response to morphine are suspected to be at least partly associated with genetic variations in the functionality or density of MOR1.

Recently we have experienced two cases of cancer in which the clinical responses to morphine therapy were completely different (i.e., sensitive and low responders). The aim of the present study was to characterize the genetic structure of two morphine-related genes, UGT2B7 and MOR1, in these two patients. Before the characterization, we attempted to identify polymorphic variations in the human UGT2B7 gene of Japanese individuals.
**Case Presentation**

**Patient 1.** A 78-year-old male with hypopharyngeal cancer was suffering from severe neck pain because of cervical lymph node metastasis. Chemoradiotherapy was performed, but the response was poor in both the original tumor and the metastasis. As his neck pain remained, a morphine slow release tablet (10 mg daily) was administered for pain relief. Two days later, severe drowsiness and confusion developed. These side effects disappeared after withdrawal of the morphine slow release tablet. Serum concentrations of morphine and glucuronide could not be measured because of the short duration of the therapy. Coadministered drugs during the morphine therapy included nifedipine (80 mg/day), carbamazepine (200 mg), and fentanyl (20 mg).

**Patient 2.** A 46-year-old female, who had a Pancoast tumor with metastases in the third rib on the left side, was suffering from severe pain in the left shoulder and upper chest wall. Six courses of chemotherapy with docetaxel were performed after concurrent chemoradiation with cisplatin and etoposide. Morphine slow release tablets (990 mg daily) were further administered for pain relief and were effective. However, the dosage of morphine necessary for the relief of pain was more than 2000 mg a day because severe pain appeared gradually. Serum concentrations of morphine, M6G and M3G at 2000 mg oral administration were 382.5, 1365.0, and 23622.0 ng/ml, respectively. Still, complete pain reduction could not be achieved because of tumor growth and so an epidural catheter was inserted. Even on a dose titration of up to 2000 mg of epidural morphine daily, she still complained of mild to moderate pain. Serum concentrations of morphine, M6G and M3G at 2000 mg epidural administration were 4964.0, 13333.0, and 133650.0 ng/ml, respectively. As the dose of epidural morphine was markedly high, tolerance at the spinal level was suspected. In addition, serum concentrations of morphine for both routes were surprisingly higher than the estimated minimum effective level (25 ± 15 ng/ml) for severe cancer pain (Gourlay et al., 1984). It should be noted that throughout the morphine therapy, no side effects were observed. Coadministered with the morphine were furosemide (20 mg), spironolactone (25 mg), haloperidol (2.25 mg), and rilmazafone (2 mg).

**Materials and Methods**

**DNA Samples.** Blood samples were obtained from 2 cancer patients and 46 unrelated healthy individuals. Genomic DNA was prepared from peripheral lymphocytes by using the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). Each subject gave written informed consent to participate in the study, which was approved by the Institutional Review Board of the Clinical Pharmacology Center, Medical Co., Ltd., and Tottori University Hospital.

**Screening and Identification of Genetic Variants.** Before primers were designed for the UGT2B7 and MOR1 genes, a two-step PCR-based cloning strategy was used to generate a number of genomic fragments covering intronic regions (DNA Walking Kit; BD Biosciences Clontech, Palo Alto, CA) (Wendel and Hoehe, 1998). The primer design was based on the sequence of the 5'-flanking region and the intron/exon junction of UGT2B7 and MOR1. These primers were designed to divide the 5'-flanking region (1.5 kb for UGT2B7 and 2.6 kb for MOR1), 4 exons (exon 1, 3, 4 and 5) for the UGT2B7 gene, and all 4 exons except exon 4 for the MOR1 gene into fragments of ~300 bp, for the screening of mutations by subsequent SSCP analysis. All PCRs were carried out in a total volume of 25 μl in the presence of 100 ng of genomic DNA, 0.25 μM of each primer, 10 × PCR buffer II, 1.5 mM MgCl2, 0.2 mM of each dNTP, and 1.25 to 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). After an initial denaturation at 94°C for 5 min, 30 to 50 cycles of 1 min at 94°C, 0.5 to 1 min at 50 to 60°C and 2 min at 72°C were followed by a final extension for 5 min at 72°C. PCR products were analyzed on 3% agarose gels to check both the size and specificity of the products. To screen for mutations of the two genes, SSCP analysis was performed using the GenePhor system (Amersham Biosciences AB, Uppsala, Sweden) as described previously (Ieiri et al., 2000). All PCR products were sequenced either directly or after subcloning on an ABI 377 automatic sequencer (Applied Biosystems). The sequencing primers were those used in the PCR amplifications. Two known variants of the UGT2B7 gene, His (UGT2B7*1) and Tyr (UGT2B7*2) at residue 268, were diagnosed by PCR amplification with use of the allele-specific primers following NdeI digestion described by Bhasker et al., 2000.

**Results**

All PCR procedures developed and used in the present study to amplify the morphine-related genes were successful. In all cases, a single PCR product of predicted size was obtained and matched the sequence predicted from the published DNA. The sequences were inspected for deviations from the original, which we defined as the “reference” alleles [GenBank accession no. AJ000341 and AF024515-6 for the MOR1 gene (Wendle and Hoehe, 1998) and AF282881 for the UGT2B7 gene (Jin et al., 1993; Riedy et al., 2000)].

In the UGT2B7 gene, 12 SNPs were detected by SSCP analysis and identified by subsequent sequencing (Table 1). A G→T transversion at position 211 (exon 1) was associated with an amino acid change from Ala71 to Ser71; this variation occurred in 32.6% of volunteers as heterozygosity and 2.2% as homozygosity. Seven SNPs were located in the 5'-flanking region with an allele frequency ranging from 0.087 to 0.913. Six of these SNPs, A-1302G, T-1295C, T-1111C, G-899A, A-327G, and T-125C, and two coding SNPs in exon 2 (UGT2B7*2) and exon 4 (C1059G) occurred simultaneously; we consistently observed at these positions the homozygous combinations (A/A-T/T-G/G-A/T-T/ T/C-C/C) and (G/G-C/C-C/C-A/A-G/G-C/C-*1/*1-G/G) and the heterozygous combinations (A/G/T/T-C/T-G/A-A/G-T/C-C/C-*2/*1-G/G), suggesting a GCCAGC*1AGTTGAT*2C haplotype. The frequencies of the UGT2B7*4 (His268) and UGT2B7*2 (Tyr268) variant were 0.707 and 0.293, respectively.

Genetic variations of the two morphine-related genes in two cancer patients with very different clinical responses to morphine therapy, one was sensitive (patient 1) and the other a low responder (patient 2), are indicated in Table 2. In the UGT2B7 gene, remarkable differences in the nucleotide sequence were observed between the patients; patient 1 had reference alleles at almost SNP positions, but a rare ATTGAT*2C sequence as homozygosity, whereas patient 2 had the predominant GCCAGC*1G sequence as homozygosity. In contrast to the UGT2B7 gene, patient 2 was a heterozygous carrier for both the G-1784A and A118G alleles, whereas patient 1 had reference alleles. In the MOR1 gene, an A→G transversion at position 118 (A118G) was associated with an amino acid substitution from Asn to Asp at codon 40.

**Discussion**

Recent pharmacogenetic and pharmacogenomic studies have indicated that genetic polymorphisms in drug-metabolizing enzymes, transporters, and target or receptor proteins are responsible for the interindividual differences in the efficacy and adverse effect profiles of many drugs. Therefore, to personalize drug therapy based on individual genetic information, sequence variations in the candidate genes, which may lead to large interindividual differences in pharmacokinetic and pharmacodynamic profiles, should be well documented. In this regard, we selected two genes and attempted to characterize the genetic background of two cancer patients whose clinical responses to morphine were different.

Before characterization of the two morphine-related genes, we analyzed allelic variants of the UGT2B7 gene in a small sample size of Japanese individuals. Twelve SNPs were identified in the human
The T-125C mutation was located in the Oct-1 factor (Ishii et al., 2000). It is interesting that all subjects having the ATTGAT sequence also had the predominant promoter sequence in Japanese subjects. Furthermore, it was much lower than that of subjects carrying the GCCAGC sequence.

Table 1, the frequency of subjects carrying the ATTGAT sequence in the UGT2B7 gene with an allelic frequency ranging from 0.022 to 0.978 (Table 1). Of these SNPs, seven were located in the 5’-flanking region. Sequence analysis of nucleotides −1 to −500 bp revealed the presence of several canonical binding sites for transcription factors, such as Oct-1, pre-B-cell leukemia transcription factor 1, and CCAAT/enhancer binding protein, which may potentially be involved in the regulation of the expression of the UGT2B7 gene (Carrier et al., 1997). A recent transcription study showed that the construct generating the (−275/+57) region had the strongest activity of all constructs evaluated, and concluded that Oct-1, as well as hepatic nuclear factor 1a, may be a key factor for fine-tuning UGT2B7 expression. A T-125C mutation was located in the Oct-1 factor (Ishii et al., 2000).

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We identified one missense mutation in the coding region of the UGT2B7 gene. At position 211 (G211T), Ala71 is replaced by Ser, which results in a change from a lipophilic residue to a hydrophilic one. Since G211T was first identified in the present study, its functional effect remains unknown. Recently, Coffman et al. (2001) demonstrated that the opioid binding site in UGT2B7 is within the first 119 amino-terminal amino acids (N-terminal half of the protein). The G211T mutation is the only identified mutation within this range.

During subcloning for the exon 2 sequence, at least three clones, A735A801T802, G735T801C802, and A735T801C802, were obtained. Although T801C802 and A801T802 produce His268 (UGT2B7*1) and Tyr268 (UGT2B7*2), respectively (Bhasker et al., 2000), these observations indicate a nonspecific PCR for exon 2. High homology of the DNA sequence within the human UGT2B genes (i.e., 70–98%) may be a possible reason for this unsuccessful PCR for exon 2. High homology of the DNA sequence within the human UGT2B genes (i.e., 70–98%) may be a possible reason for this unsuccessful PCR for exon 2.

In addition to exon 2, we could not analyze the exon 6 sequence because of the same reason. Thus, we used a conventional PCR-restriction fragment length polymorphism method for the identification of two variants in exon 2.
As shown in Table 1, the frequencies of the *1 allele, patent 1 (i.e., sensitive to morphine) had the rare metabolic route (i.e., morphine 6-glucuronidation) cannot be dis- 
differ. However, a significant effect on specific substrates and/or 
substrates (androsterone, menthol and morphine 3-glucuronidation) 
good accord with previous findings (Bhasker et al., 2000). Bhasker et 
concentration curve ratios of M6G and M3G to morphine were 1.4 to 
edural morphine treatment of cancer patients, plasma area under the 
were surprisingly different between the two patients. During oral or 
susceptibility remains unknown. Additional genotype-phenotype 
clinical use of morphine for analgesia.

References


Carrier JS, Turregon D, Journault K, Hum DW, and Belanger A (2000) Isolation and character- 


Ishii Y, Hansen AJ, and Mackenzie PI (2000) Octamer transcription factor-1 enhances hepatic glucuronidation of morphine-related genes in two cancer patients whose clinical responses to morphine were very different (case 1 was sensitive, whereas case 2 was a possible low responder).

As shown in Table 1, the frequencies of the UG2T2B7*1 and UG2T2B7*2 variants were 0.707 and 0.293, respectively, and these values were in good accord with previous findings (Bhasker et al., 2000). Bhasker et al. (2000) have investigated the functional significance of the two variants by determining rates of glucuronidation of selected UG2T2B7 substrates (androsterone, menthol and morphine 3-glucuronidation) by microsomes from a panel of genotyped human livers. Although there was a trend toward a lower glucuronidation capability for the UG2T2B7*2 homozygous livers, intergenotype differences were not significant for any of the substrates (Bhasker et al., 2000). Coffman et al. (1998) also reported that glucuronidation kinetics of the three substrates by cDNA-expressed UG2T2B7*1 and UG2T2B7*2 did not differ. However, a significant effect on specific substrates and/or metabolic route (i.e., morphine 6-glucuronidation) cannot be dis- counted. Interestingly, in contrast to patient 2 who was a homozygote for the *1 allele, patient 1 (i.e., sensitive to morphine) had the rare *2/*2 genotype.

The present study describes substantial differences in genotype patterns between patients with different clinical responses to mor- phine therapy (Table 2). In the MOR1 gene, an A118G missense mutation (Asn40Asp) was observed in patient 2 who seemed to be a 
low responder. A118G is the most frequent mutation occurring at an 
allelic frequency of 10 to 20% depending on the population evaluated, 
and several association studies (e.g., alcohol or opioid dependence and 
epilepsy) have been conducted on it (Bergen et al., 1997; Bond et al., 1998; Sander et al., 2000). The results, however, were controversial. 
Still, the existence of A118G in a possible low responder (i.e., large 
amounts of morphine needed to control pain) was consistent with the 
findings of Caraco et al. (2001) who demonstrated that Asp40 carriers require a significantly higher dose of alfentanil as compared with 
Asn40 carriers to control the analgesic effect during extracorporeal 
shock wave lithotripsy for kidney calculi.

As shown in Table 2, the genotype patterns of the UG2T2B7 gene 
were surprisingly different between the two patients. During oral or 
epidural morphine treatment of cancer patients, plasma area under the 
concentration curve ratios of M6G and M3G to morphine were 1.4 to 
9.7:1 and 7.9 to 55.8:1, respectively (Sawa et al., 1983; Osborne et al., 
1990). In patient 2, the serum level ratio of M6G to morphine was 
3.9:1 and of M3G to morphine was 61.7:1 after oral administration, 
and the respective values for the epidural administration were 2.7:1 
and 26.9:1. Since serum level ratios in patient 2 were comparable with 
the plasma area under the concentration curve ratios, it is suggested 
that the predominant GCCAGC*1G sequence is not directly associ- 
ated with the "poor metabolizer" phenotype.

In this preliminary study, we attempted the systematic analysis of 
variations in two candidate gene sequences expected to be of im- 
portance in personalized morphine therapy. However, the functional 
properties of the SNPs in large groups of patients and controls, as well as in 
vitro studies are needed to establish appropriate methods for 
clinical use of morphine for analgesia.

| Table 2 |
| Sequence variability of morphine-related genes in two cancer patients whose clinical responses to morphine were very different (case 1 was sensitive, whereas case 2 was a possible low responder). |
| Gene | Location | Position | Patient | Case 1 | Case 2 |
| UG2T2B7 | promoter | −1302 | A/A | G/G | |
| | | −1295 | T/T | C/C | |
| | | −1111 | T/T | C/C | |
| | | −899 | G/G | A/A | |
| | | −327 | A/A | G/G | |
| | | −161 | T/T | C/T | |
| | | −125 | T/T | C/C | |
| exon 1 | 211 | G/G | G/T | |
| | 372 | A/A | A/G | |
| exon 2 | 801, 802 | (AT)/(AT) | (TC)/(TC) | |
| exon 4 | 1059 | C/C | G/G | |
| | 1062 | C/C | T/T | |
| MOR1 | promoter | −1784 | G/G | G/G | |
| | 118 | A/A | A/G | |