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ORIGINAL ARTICLE

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Six novel UDP-glucuronosyltransferase (UGT1A3) polymorphisms with varying activity

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Abstract Human UDP-glucuronosyltransferase (UGT) is a part of a major excretion pathway for endobiotics and xenobiotics. The *UGT* family of genes is highly polymorphic, and our aim is to describe novel polymorphisms at the *UGT1A3* locus and determine how they alter substrate metabolism and drug reactions. One hundred healthy Japanese adults volunteered for the present study. We sequenced PCR-amplified fragments of the gene directly, and calculated the frequency of the genetic variations detected. To measure variant enzyme activity, we constructed five expression models and used estrone as the substrate in the assays. We identified six novel single nucleotide polymorphisms (SNPs). Of these, four caused amino acid substitutions (17A → G: Q6R, 31T → C: W11R, 133C → T: R45W, and 140T → C: V47A) and the remaining two were silent (81G → A: E27E and 447A → G: A159A). We found five types of alleles having differing SNP combinations: wild type (frequency = 0.61), W11R-E27E-A159A (0.10), Q6A-W11R-E27E-A159A (0.055), W11R-E27E-V47A-A159A (0.125), and R45W (0.11). Expression studies found that the variants changed the enzyme efficiencies (K_m/V_{max}) to 121% of the wild type for W11R, 86% for Q6R-W11R, 369% for W11R-V47A, and 70% for R45W. Several UGT 1A3 polymorphisms exist in the Japanese population, having different levels of activity. These polymorphisms are capable of affecting the steady state levels of estrogens, and may increase sensitivity to adverse drug effects.

Keywords UDP-glucuronosyltransferase · UGT1A3 · Polymorphism · Drug metabolism · Estrone

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Introduction

Glucuronidation catalyzed by UDP-glucuronosyltransferases (UGTs) is a part of a major excretion pathway for lipophilic endobiotics and xenobiotics. Based on their amino acid sequence similarities, UGTs are divided into two families, UGT1 and UGT2 (Mackenzie et al. 1997). The UGT1 gene is unique in being found with 13 different exons 1 (*UGT1A1* to *UGT1A13P*), whereas exons 2–5 are common to all mRNAs expressed by the gene. The UGT1 family contains four pseudogenes (exons 1: *UGT1ABP*, *UGT1A11P*, *UGT1A12P*, and *UGT1A13P*). UGT1 mRNAs are processed by differential splicing. Gene products of the *UGT2* family, by contrast, are transcribed from individual genes (Mackenzie and Rodbourn 1990; Haque et al. 1991). Each isoform has unique substrate and tissue specificities, and the *UGT* gene family is highly polymorphic. We have earlier reported mutations and polymorphisms in *UGT1A1* that are responsible for Gilbert's syndrome, neonatal hyperbilirubinemia, and breast milk jaundice (Sato et al. 1996; Maruo et al. 1999, 2000), and other researchers have reported a role for a UGT1A1 polymorphism in the adverse effects (such as diarrhea and bone marrow suppression) of the antitumor agent irinotecan (Ando et al. 2000). Irinotecan is a prodrug, and is metabolized to an active form (SN-38) which is inactivated by glucuronidation by UGT1A1 and UGT1A7. Polymorphisms have also been found in

UGT1A6, *UGT1A7*, *UGT1A8*, *UGT2B4*, *UGT2B7*, and *UGT2B15*. Many of these affect functional properties of the coded proteins (Lampe et al. 1999; Ciotti et al. 1997; Guillemette et al. 2000a; Vogel et al. 2001; Huang et al. 2002; Lavesque et al. 1999; Bhasker et al. 2000; Macleod et al. 2000).

UGT1A3 is expressed in the liver, the biliary and gastric tissue and the intestine (Strassburg et al. 1997; Mojarrabi et al. 1996). It catalyzes the glucuronidation of important endogenous substances as well as many commonly prescribed drugs: estrone, 2-hydroxyestrone, primary amine, tertiary amines, hydroxylated benzo[a]pyrene metabolites, 2-acetylaminofluorene metabolites, flavonoids, 7-hydroxycoumarins, opioids, and anthraquinones (Mojarrabi et al. 1996; Green et al. 1998). The type and incidence of UGT polymorphisms differ between races (Maruo et al. 1999; Monaghan et al. 1996; Bosma et al. 1995; Akaba et al. 1998). Here, we have analyzed the type and incidence of UGT1A3 polymorphisms in a Japanese population, correlating type with activity.

Materials and methods

Sequence analysis of *UGT1A3*

Genomic DNA was isolated from the leukocytes of 100 healthy Japanese volunteers who had given informed consent. The study was approved by the ethics committee of Shiga University of Medical Science. Exon 1 of *UGT1A3* was amplified by PCR from genomic DNA with primer pair 5′-CACGTTGATTTGCTAAGTGG-3′/5′-TGGATGAAGGCACCAATACA-3′. The forward primer was located upstream of the initiation codon, and the reverse primer was located in intron 1. The 978-bp product was amplified by PCR under the following conditions: initial denaturing for 2 min at 94°C, followed by 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C for 32 cycles with the PCR thermal cycler PERSONAL (TaKaRa, Kyoto, Japan). A final extension for 8 min at 72°C ensured complete extension of the PCR products. The amplified DNA fragment sequences were determined directly with a dRhodamine terminator FS Ready Reaction Kit and PRISM 310 (Applied Biosystems, Foster City, CA, USA). The sequencing primers were as follows: 5′-TGAAGAAAGCAAATGTAGC-3′/5′-ACCTATGCCATTTCGTGGAC-3′/5′-TTGAGTGTGGCCCAGCACAT-3′/5′-TAGACTTTAAGGGCACACAG-3′/5′-GGAGCAGAAAAAGCATGGCA-3′/5′-TGGGGTGAGGACCACTG-3′.

To determine *cis* vs *trans* arrangements of the mutations on homologous chromosomes, we subcloned PCR products containing UGT1A3 exon 1 to pCR 2.1 vectors using a TA-cloning kit (Invitrogen, San Diego, CA, USA). PCR fragments (30 ng) that included exon 1 were ligated with 50 ng of the vector. Transformation by the ligated products was performed with a Competent High JM109 (Toyobo, Osaka, Japan).

Construction of expression vectors

cDNA of wild type *UGT1A3* from a human cDNA library (TaKaRa) was amplified by PCR with the primer pair 5′-TGTCTTCTGCTGAGATGGCCAC-3′/5′-GAATCCCGCACTCCCAAACAGG-3′, and was inserted into a pCR3.1 expression vector using a eukaryotic bidirectional TA cloning kit (Invitrogen). Mutations were induced by site-directed mutagenesis using a Mutan Km Kit (TaKaRa) according to the manufacturer's instructions. The constructed cDNA was cut out from the pCR3.1 vector with two restriction enzymes (*Hind* III and *Xba* I) and ligated into a pkF18 vector (TaKaRa) for mutagenesis. We used the following primers to introduce the nucleotide substitutions (the changed nucleotides are underlined): 5′-ACAGGACTCCGGGTTCCCCTG-3′ for 17A → G (Q6R), 5′-TCCCCTGCCGCGGCTGGCCAC-3′ for 31T → C (W11R), 5′-GCTCAGCATGTGGGAGGTCTT-3′ for 133C → T (R45W), 5′-ATGCGGGAGGCCTTGCGGGAG-3′ for 140T → C (V47A). After the substitutions had been introduced into the pkF18 vectors, the mutated cDNAs were cut out and re-ligated into the pCR3.1 vector. The substitution sites and other parts of the *UGT1A3* cDNA were checked by sequencing. Four expression vectors of *UGT1A3* cDNA with different types of substitutions were constructed; two were single substitution models of W11R and R45W, and two were double substitution models of Q6R-W11R and W11R-V47A.

Transfection of UGT1A3-expression plasmid into COS-7 cells

COS-7 cells were suspended in Dulbecco's modified Eagle medium with 10% fetal bovine serum and seeded at 6×10^5 onto 100-mm culture plates. Twenty-four hours later, 3 μg plasmid DNA was transfected into these COS-7 cells with Gene PORTER transfection reagent (Gene Therapy Systems, San Diego, CA, USA). After 48 h the transfected cells were harvested and stored at −80°C prior to the enzyme assay.

Assay of UGT1A3 activity

UGT activity was assayed under linear conditions with time and protein as described previously (Yamamoto et al. 1998; Bansal and Gessner 1980; Ito et al. 2001), apart from a minor modification. The incubation mixture contained: cell homogenate sonicated for 30 s, unconjugated estrone (3.125–400 μM), UDP-glucuronic acid (500 μM), 0.25 μCi (9.25 kBq) [^{14}C]UDP-glucuronic acid (8.75 μM), DMSO (1%), $MgCl_2$ (10 mM), and 100 mM Tris–maleate buffer (pH 7.4) in a final volume of 100 μl. Incubation was carried out at 37°C for 10 min. The resulting estrone–glucuronide was isolated by thin layer chromatography (TLC) on TLC plastic sheet 5748 (Merck, Darmstadt). The TLC plates were scanned on an Instant Imager (Packard, Meriden, CT, USA).

The velocity of the UGT1A3 reaction is proportional to the amount of expressed cell homogenate present at up to 750 μg (Fig. 1). When we measured the reaction product (estrone–glucuronide) formed over time at 150 μg, we found linearity for

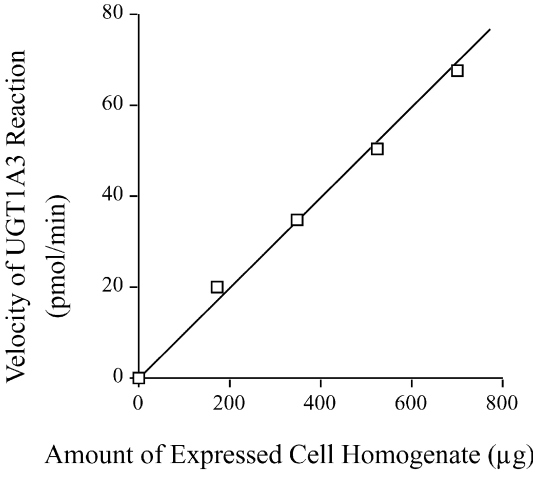

Fig. 1 Correlation of the velocity of the UGT1A3 reaction with the amount of enzyme in the reaction mixture

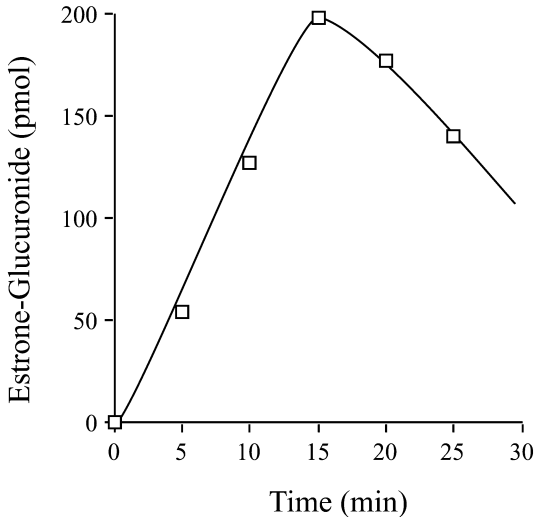

Fig. 2 Time course of the UGT1A3 reaction. The reaction mixture contained 150 μg of expressed cell homogenate

15 min (Fig. 2). We carried out subsequent incubations for 10 min. When calculating the activity of the various polymorphic forms of UGT1A3, we made adjustments for the amount of UGT1A3 protein present. Expression experiments were carried out independently in triplicate and the means taken.

Preparation of antibody and measurement of expressed protein

We synthesized a 15-amino-acid segment: (CE-VNMHIKEENFFTL) of UGT1A3, and generated a polyclonal antibody against it by immunizing rabbits. Immunoaffinity antibody purification was carried out by affinity chromatography of agarose gel coupled with 6 mg of the synthesized segment. The final titer of the purified antibody was 1:51,200.

Western blot analysis

Cell homogenates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein was transferred to a PVDF membrane (BIO-RAD, Hercules, CA, USA) and visualized with ECL Plus Western Blotting Detection Regents (Amersham Biosciences, Buckinghamshire, UK). The membrane was incubated for 1 h in blocking solution, 1 h in a solution of anti-UGT1A3 antibody (1:500), and 1 h in a solution of anti-rabbit antibody (1:10,000). The detection solution was added and the membrane was exposed to a film for 15 min. The relative amounts of UGT1A3 expressed at the protein band peaks were measured with an Image Master-CL (Amersham Biosciences, Uppsala, Sweden).

A 55-kDa protein band was detected in all expression models but not in the mock transfection, and the molecular weight of the band was similar to the value reported previously [14]. The relative amounts of mutated UGT1A3s were within 0.9–1.1 times that of normal UGT1A3.

Data analysis

We calculated kinetic parameters using the Prism 3.0 software (Graph Pad Software, Inc., San Diego, CA, USA) using non-linear regression on the Michaelis–Menten equation. All data shown are the results from three separate experiments. The efficiencies for the different alleles were studied by analysis of variance and Schiff's test for pairwise comparisons.

Table 1 Single nucleotide polymorphisms identified in exon 1 of *UGT1A3*

Nucleotide position	Wild type	Mutant	Amino acid change	SNP frequency ($n = 200$)
17	A	G	Q6R	0.055
31	T	C	W11R	0.28
81	G	A	E27E (silent)	0.28
133	C	T	R45W	0.11
140	T	C	V47A	0.125
477	A	G	A159A (silent)	0.28

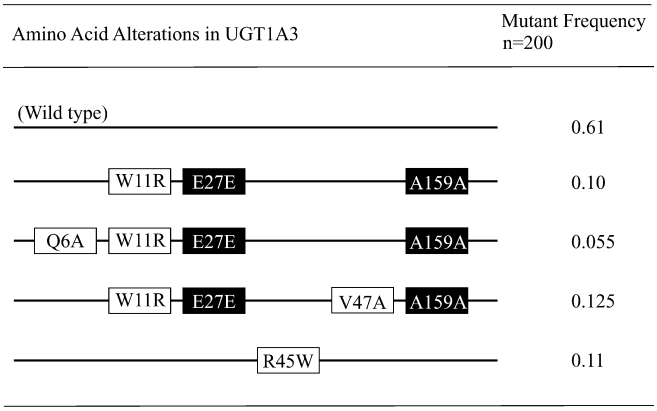

Fig. 3 Amino acid substitutions in UGT1A3 coded by allelic polymorphisms, and their frequencies

Results

Identification and frequency of SNPs (single nucleotide polymorphisms) in *UGT1A3*

We identified six novel nucleotide substitutions in exon 1 of *UGT1A3*, of which four caused amino acid substitutions (Table 1). The incidence of the substitutions ranged from 0.055 to 0.28. The frequency of each of the substitutions was greater than 0.01, indicating that they represent nucleotide sequence polymorphisms in the sampled population of Japanese. The incidences of the polymorphisms indicate the existence of alleles containing different SNP combinations. Indeed, analysis of the subcloned *UGT1A3* PCR products revealed the presence of five alleles with frequencies ranging from 0.055 (for an allele with W11R, E27E, V47A and A159A) to 0.61 (for the wild type) (Fig. 3). No additional SNPs were detected in exons 2–5 of UGT1A3 in all alleles.

Activities of UGT1A3 isoforms

The glucuronidation of estrone by wild type UGT1A3 reached a maximum at about 25 μM estrone and then declined (Fig. 4), indicating substrate inhibition of the enzyme. Table 2 shows the apparent K_m, V_{max}, and

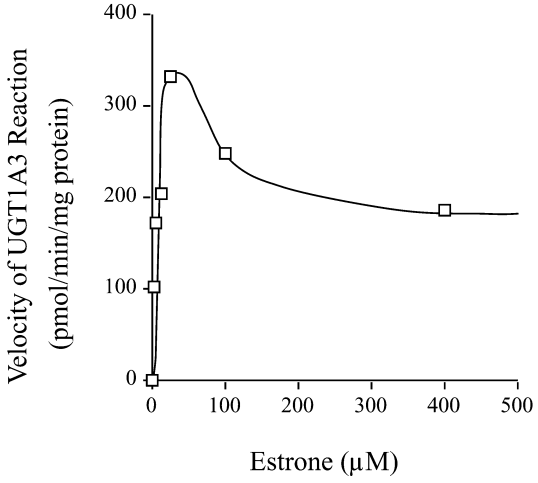

Fig. 4 Correlation between the velocity of the UGT1A3 reaction and estrone concentration

relative efficiency (V_{max}/K_m) of the expressed wild type and four mutant enzymes at this maximum of 25 μM substrate concentration. The efficiency of the homozygous models of the four variant alleles relative to the wild type are as follows: W11R (a model of W11R-E27E-A159A), 121%; Q6R-W11R (Q6A-W11R-E27E-A159A), 86%; W11R-V47A (W11R-E27E-V47A-A159A), 369%; and R45W, 70%. Analysis of variance revealed significant differences among the five alleles (degrees of freedom: 4/10, $F=9.548$, $P=0.0019$). The efficiency of the W11R-V47A was significantly higher than that of the wild type ($P=0.0126$), the W11R ($P=0.0208$), the Q6R-W11R ($P=0.0090$), and the R45W ($P=0.0062$).

Discussion

UGTs have a crucial role in the detoxification of xenobiotic and endobiotic substances by glucuronidation. UGT defects cause diseases, and a defect that reduces the enzyme's activity increases the risk of adverse drug effects (Maruo et al. 1999; Yamamoto et al. 1998; Ando et al. 2000). Mutations of *UGT1A1*, which codes for bilirubin UGT, causes the hereditary unconjugated hyperbilirubinemias (type I and II Crigler–Najjar syndrome and Gilbert's syndrome) (Adachi et al. 1996). *UGT1A1*polymorphisms may also be involved in the toxicity of the anticancer drug irinotecan, in inducing diarrhea and suppressing bone marrow (Ando et al. 2000), and may increase the risk of breast cancer through reduced glucuronidation of estradiol (Guillemette et al. 2000b). UGT1A7 polymorphisms that reduce the glucuronidation of benzo[a]pyrene accelerate the rate of smoking-induced orolaryngeal cancer (Zheng et al. 2001). In the present study we have identified polymorphisms of UGT1A3, an enzyme that glucuronidates xenobiotic and endobiotic substances, and we find that exon 1 of *UGT1A3* prepared from the DNA of 100 healthy Japanese volunteers contained six SNPs. Four of these resulted in amino acid substitutions (Q6R, W11R, W45R, and V47A) and two were silent substitutions at codons 27 and 159.

The incidences of SNPs indicate the presence of polymorphisms, some having a combination of SNPs. Nucleotide sequencing revealed five polymorphisms that coded for five types of UGT1A3 protein in the population sample studied (Fig. 3). The most frequent wild type allele reported for *UGT1A3* encodes an enzyme with Q6-W11-R45-V47 (Ritter et al. 1992a). In the present study, about one third (36%) of the population was homozygous for wild type UGT1A3. We found two pairs of concurrent SNPs which cause amino acid substitutions on single alleles; these are Q6A-W11R and W11R-V47A.

We have previously reported that the activity of the UGT1A1 polymorphism G71R is 32% of the wild type (Yamamoto et al. 1998). This is the mutation responsible for Gilbert's syndrome, neonatal hyperbilirubinemia, and breast milk jaundice (Sato et al. 1996; Maruo et al. 1999, 2000). Other UGT1 isoforms that show reduced drug metabolism have also been reported (Ando et al. 2000; Ciotti et al. 1997; Guillemette et al. 2000a; Adachi et al. 1996). Indeed, all the mutant UGTs reported previously showed reduced activity (Yamamoto et al. 1998; Ueyama et al. 1997). In the present study, however, the variant UGT1A3 showed increased activity, with a relative efficiency of 370% (for W11R-V47A) (Table 2).

Table 2 Kinetic parameters of wild type and mutant UGT1A3. Assays were performed as outlined in Materials and Methods using 500 μM UDPGA. COS-7 extracts using 150 μg protein for each assay in reactions that were incubated for 10 min at 37°C. Data are shown as mean ± SEM of three independent experiments

Allele	V_{max}[a] (pmol/min/mg)	K_m[b] (μM)	V_{max}/K_m (μM/min/mg)	% of wild type (%)
Wild type	263.8 ± 36.8	7.392 ± 2.113	39.61 ± 7.29	100
W11R	244.3 ± 61.0	8.432 ± 3.734	47.93 ± 21.87	121
Q6R-W11R	307.2 ± 70.2	9.359 ± 2.766	33.99 ± 1.97	86
W11R-V47A	231.4 ± 14.7	1.683 ± 0.338	146.3 ± 24.2	369[c]
R45W	191.3 ± 58.6	10.84 ± 4.45	27.66 ± 12.27	70

[a] V_{max}maximum velocity
[b] K_mMichaelis constant
[c] Significant difference from the wild type ($P=0.0126$). *P*value was obtained by using Scheff's test

The 20 amino acid residues of the N-terminal of UGT1A1 comprise a signal peptide (Iyanagi et al. 1986; Seppen et al. 1996). The N-terminal region of UGT1A3 is similarly constructed, and may also be a signal peptide. L15R in the signal peptide of UGT1A1 leads to a loss of enzyme activity, due to impaired interaction between the mutant protein and the endoplasmic reticulum (ER) translocation machinery, and carriers suffer from Crigler–Najjar syndrome type II (Seppen et al. 1996). In our study of UGT1A3, however, two amino acid substitutions (W11R and Q6R) are located on the probable signal peptide, and the activities of enzymes with these substitutions are 121 and 86% of the wild type (Table 2). These two substitutions do not therefore seem to affect the targeting of the variant proteins to the ER. R45W decreased the enzyme activity to 70% of the wild type. However, enzyme activity of the two-residue amino acid substitutions W11R-V47A, to our surprise, was 3.7 times that of the wild type. It is possible that amino acid residues around 45–47 are important for activity. The presence polymorphic enzymes with decreased or increased activity at frequencies of about 10% in the Japanese population may be important for steroid female hormone- dependent diseases as well as for adverse drug effects.

UGTs represent an important class of phase II detoxification enzymes that catalyze the transfer of glucuronic acid to many substrates in the liver and extrahepatic tissues (Dutton 1980). Compounds inactivated by glucuronic acid conjugation include steroid hormones, such as estrogens and catechol estrogens (Cheng et al. 1990; Ritter et al. 1990, 1992b; Albert et al. 1999). UGT enzymes may therefore assist in the maintenance of steady-state levels of steroids in those tissues (Hum et al. 1999; Belanger et al. 1998). Estrone is metabolized by UGT1A3, 1A8, and 1A9, and is an endogenous female hormone. Although estrogens are required for growth and development of target tissues, they also increase the risk of uterine and breast cancers when the endogenous estrogen concentration is increased in target tissues (Henderson et al. 1982; McGonigle et al. 1994; Bernstein and Ross 1993; Toniolo et al. 1995; Adlercreutz et al. 1994). Serum estrone is also associated with risks for osteoporosis and colon carcinoma (Ohta et al. 1993; English et al. 1999), and the different glucuronidation rates shown by each allele in in vitro expression studies of UGT1A3 suggest that the allelic variation contribute to estrone-associated clinical conditions. Moreover, UGT1A3 catalyzes the glucuronidation of many commonly used drugs [13], so that combinations of different types of *UGT1A3* alleles might contribute to individual differences of drug metabolism and susceptibility to side effects.

In conclusion, we detected six novel SNPs in *UGT1A3*. Combinations of the SNPs generated five types of alleles. Carriers of the alleles have different levels of enzyme activity ranging from 70 to 369% of the wild type. These results suggest that UGT1A3 polymorphism is partly responsible for individual differences in drug sensitivities, and susceptibility to diseases that relate to estrogen levels.

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