A pharmacogenetics study of the human glucuronosyltransferase UGT1A4

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Running head: Genetic polymorphisms of *UGT1A4*

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ABSTRACT

UGT1A4 is primarily expressed in the liver and exhibits catalytic activities for various drugs. Amongst the few UGT1A4 polymorphisms evaluated, studies support the alteration of UGT1A4-mediated glucuronidation by a few variations including the $Pro^{24}Thr$ and $Leu^{48}Val$ variants (referred to as UGT1A4*2 and *3). We therefore investigated genetic mechanisms that might contribute to interindividual variation in UGT1A4 expression and activity. The *UGT1A4* gene was sequenced from -4963 bp relative to the ATG to 2000 bp after the first exon in 184 unrelated Caucasians and African-Americans. We identified a large number of genetic variations, including 13 intronic, 39 promoter, as well as 14 exonic polymorphisms, with 10 that lead to amino-acid changes. Of the nucleotide variations found in the -5kb promoter region, 5 are located in the proximal region (first 500 bp), and positioned in putative HNF-1 and OCT-1 binding sites. Four of these variants, placed at -163, -219, -419 and -463, are in complete linkage disequilibrium with the $Leu^{48}Val$ coding region variant and with several variants in the upstream region of the promoter. Transient transfections of reference and variant promoter constructs (from position -500 to +1) in different cell lines with or without co-expression of HNF-1 and/or OCT-1, demonstrated limited effect of these variations. However, several coding variants significantly modified the enzyme kinetics for tamoxifen and Z-4-hydroxytamoxifen (Val^{48}, Asp^{50}, Gln^{56}, Phe^{176}, Asn^{250}, Leu^{276}). Our results reveal that, despite a large number of polymorphisms located in the promoter region, the exonic variants are those expected to have a potential *in vivo* effect.

Keywords: pharmacogenetics, polymorphisms, tamoxifen, UGT

INTRODUCTION

UDP-glucuronosyltransferase (UGT) enzymes comprise a superfamily of membrane proteins that catalyze the glucuronidation of a wide range of xenobiotics and endogenous compounds. As a major pathway the among phase II reactions in drug metabolism, interindividual variation in the glucuronidation pathway has received much attention and a number of genetic variations has been described, some of which have a clear pharmacological impact. To this day, nineteen functional proteins have been described, classified into three subfamilies: UGT1A, UGT2A and UGT2B [1-3]. The subfamily UGT1 includes nine active UGT isoforms encoded by a single gene *UGT1* than spans 200kb. The UGT1A isoforms are encoded by four shared exons (2-5) that code for the carboxy terminal region of the protein, and variable exons 1, which corresponds to the substrate binding domain and provides differential substrate specificity [4].

UGT1A4, together with UGT1A3, is the main enzyme responsible for addition of glucuronic acid to amino groups (N-glucuronidation) [5,6]. The UGT1A4 protein is primarily expressed in the liver and in bile ducts, colon and small intestine. This enzyme exhibits catalytic activities mostly for primary and secondary amines, present in various therapeutic drugs [7]. Among these are a number of psychiatric drugs, including tricyclic antidepressants, antipsychotics and anticonvulsants. Trifluoperazine (TFP) is known to be readily and specifically glucuronidated by UGT1A4 [8]. The UGT1A4 enzyme was also reported to have the capacity to generate one of the glucuronides of the dietary carcinogen N-OH-PhIP found in cooked meat [9,10]. Tamoxifen, a nonsteroidal

antiestrogen widely used for chemotherapy of hormone-dependent breast cancer, was also reported as a substrate for this enzyme [11]. Genetic variability in tamoxifen metabolism is one putative causal factor associated with interpatient variability in response to therapy [12]. Tamoxifen can be converted into several oxidative compounds [13], including 4-OH-tamoxifen, which is considered as its active metabolite, and both are shown to be metabolized by UGT1A4 into ammonium-linked glucuronides [11,12,14].

Frequency of genetic variations and haplotypes in *UGT1A4* has been described for the Caucasian and Japanese populations, however, these studies included limited analysis of the promoter and intronic regions [15-17]. A careful evaluation of the function of *UGT1A4* coding region polymorphisms has been limited to the $P^{24}T$ and $L^{48}V$ variants [15,18]. In contrast, only two promoter variations (-163G>A and -219C>T) were evaluated *in vitro* in a single study [19].

The main objective of this study was 1) to screen the *UGT1A4* gene for polymorphisms in the promoter and coding region, from -4962bp relative to the transcription start site to 2000bp after the first exon, and, 2) to establish their potential effect on UGT1A4 enzymatic function and expression *in vitro*.

METHODS

Materials

Methanol, acetonitrile and formic acid were purchased from VWR (Montreal, Canada). Ammonium formate was purchased from Laboratoire Mat (Quebec, Canada). Tamoxifen, z-4-hydroxytamoxifen, β-glucuronidase type VII from *E. Coli* and UDP-glucuronic acid were obtained from Sigma-Aldrich (St. Louis, MO). N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP), d_5-tamoxifen and d_5-4-hydroxytamoxifen were purchased from Toronto Research Chemical (Toronto, ON, Canada). Tamoxifen glucuronide, d_5-tamoxifen glucuronide, z-4-hydroxytamoxifen glucuronide and d_5-z-4-hydroxytamoxifen were obtained from enzymatic assays using liver microsomes. Human cell lines, HEK293, ACHN, HepG2 and Caco-2, were obtained from the American Type Culture Collection (Rockville, MD). All other chemicals and reagents were of the highest grade commercially available.

DNA samples from 100 unrelated Caucasians and from 36 Caucasian/African-American were described from previous studies [20,21]. Liver microsomes, DNA and RNA from American subjects were used as described previously [22,23]. All subjects provided written consent for experimental purposes, and the Institutional Review Boards approved the use of these samples.

UGT1A4 sequencing

PCR was used to amplify the *UGT1A4* gene from -4962bp in the promoter region to 2000 bp after the first exon in 100 Caucasian individuals. Sequencing of the promoter (-2500bp) and exonic region was also performed for the 52 liver samples. The first exon was also sequenced in 36 African-American individuals. Amplification and sequencing primers are shown in Table 1. PCR conditions were 95°C for 1 min for initial denaturation, followed by 40 cycles at 95°C for 30 sec, 55-65°C for 30-40 sec and 72°C for 1-1.5 min, with a final extension at 72°C for 7 min. Specificity of primers was confirmed by direct sequencing of all PCR products. Amplicons were sequenced with an ABI 3700 automated sequencer using Big Dye (PerkinElmer Life Sciences, Boston, MA) dye primer chemistry. Samples were sequenced on both strands with primers listed in Table 1. Samples with ambiguous sequencing chromatograms were subjected to a second, independent amplification, followed by DNA sequencing. Sequences were analyzed with Staden preGap4 and Gap4 programs (Open Source Technology Group, CA, USA, http://staden.sourceforge.net/) and compared with the reference sequence (GenBank AF297093) to assess genetic variations. Linkage disequilibrium analysis and haplotype determination were performed with the Haploview 3.32 software (www.broad.mit.edu/mpg/haploview).

UGT1A4 variants and microsomal preparations

UGT1A4 variant alleles were generated by site-directed mutagenesis using the pcDNA3 vector, using a procedure described previously [24,25]. Stable HEK293 cell transfection with variant pcDNA3-UGT expression plasmids, preparation of microsomes

by differential centrifugation, and assessment of UGT protein levels by Western blot have been previously described [24-26].

Transient transfections of *UGT1A4* promoter constructs

Reference and variant promoter constructs of 606 bp were isolated by PCR from carriers of reference (-163G, -219C, -419G and -457C) or variant promoter (-163A, -219T, -419A and -457T). Additional variant alleles were generated by site-directed mutagenesis using the pcDNA3 vector. Promoter constructs of 500 bp were cloned upstream of the luciferase reporter gene in the PGL3 basic vector (Promega, Madison, WI) and transient transfections were performed using Lipofectamine2000 as the transfectant (Invitrogen, Burlington, ON). Reference and variant *UGT1A4* promoter constructs (500ng) were transfected in HepG2, Caco-2 and ACHN cell lines in the presence or absence of 25ng HNF-1 and/or 25ng OCT-1 (in pcDNA1.1 vector) transcription factors. Addition of pRL-null (5ng) and the pBS-SK+ empty vector yielded a total of 555ng of DNA for each transfection. Luciferase activity was assessed with the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) following the manufacturer's protocol. Luciferase activity was measured with 30 µL of cell lysate in a 96-well plate on an LB96V microplate luminometer (EG&G Berthold, Bad Wildbad, Germany). Results were obtained by comparing luciferase in transfected cells to the basal level ratio in cell lines transfected with an empty pGL3 vector (fold over pGL3).

Enzymatic assays with UGT1A4 substrates: tamoxifen, trans-4-hydroxy-tamoxifen, trifluoperazine and N-OH-PhIP

Enzymatic assays were performed according to a standard procedure [26,27]. Briefly, assays consisted of 1h incubations at 37°C with 5 to 50 μg of microsomal preparation according to the linearity of the reaction, 50 mM Tris-HCl, 10 mM MgCl2, 2 mM UDP-glucuronic acid, leupeptin, pepstatin and phosphatidylcholine. Assays were terminated by adding 100μL ACN + 0.000002% (v/v) butylated hydroxytoluene (BHT) for tamoxifen or 100μL MeOH + 0.000002% BHT for 4-hydroxytamoxifen. For glucuronidation experiments in liver samples, a fixed concentration of either N-OH-PhIP (25 μM) or trifluoperazine (200 μM) was used. In kinetic analysis experiments, varying tamoxifen (1-100μM) and Z-4-hydroxytamoxifen (1-500μM) concentrations were used. The procedure for enzymatic assays with N-OH-PhIP and trifluoperazine was described previously [8,28]. Statistical evaluation of best-fit model was used to select the enzyme kinetic model and confirmed by a visual inspection of fitted functions (V as a function of [S]) and Eadie-Hofstee plots (V as a function of V/[S]). Kinetic parameter calculations were performed with Sigma Plot 8.0 software assisted by Enzyme Kinetics 1.1 software (SPSS Inc., Chicago, IL). Values are expressed as the mean of at least two experiments performed at least in duplicate.

LC/MS analysis of glucuronide products

Detection of UGT1A4-generated N-OH-PhIP and TFP glucuronides was performed as described previously [8,10]. For the detection of tamoxifen, 4-hydroxytamoxifen glucuronides, the LC-MS/MS system consisted of a mass spectrometer (model API 3200, Concord, Canada) operated in multiple reactions monitoring mode

(MRM) and equipped with an electrospray ionization interface in positive ion mode. Positive-product MRM ions pairs were m/z 548.3 → 372.1 for tamoxifen-glucuronide, m/z 553.3 → 377.1 for d_5-tamoxifen-glucuronide, m/z 564.3 → 388.3 for Z-OH-tamoxifen-glucuronide and m/z 569.0 → 393.0 for d_5-Z-OH-tamoxifen- glucuronide. The ion source temperature was set at 450°C. The ion spray voltage, declusting potential and entrance potential were set at 5000, 15 and 12V respectively. The collision energy for tamoxifen glucuronide and Z-OH-tamoxifen-glucuronide were –42 V. The chromatographic system consisted of an Agilent 1200 series (Agilent, Palo Alto, CA, USA). For tamoxifen glucuronide, the chromatographic separation was achieved with a Gemini C_6 column with 3 μm packing material, 100 X 4.6 mm (Phenomenex). The mobiles phases were (solvent A) water 0,1% formic acid (v/v) and (solvent B) acetonitrile 0,1% acid formic (v/v). Separation was achieved using a linear gradient of 45-60% B in 2.0min at a flow rate of 0.9 ml/min. Then, the column was flushed with 95%B for 2 minutes and re-equilibrated to initial conditions over 3 min. For the Z-OH-tamoxifen glucuronide, the chromatographic separation was achieved with a ACE-3 HL column with 3 μm packing material, 100 X 4.6 mm (Life Science, Peterborough, Canada). The mobiles phases were (solvent A) water 0,4% formic acid (v/v) and (solvent B) methanol. Separation was achieved using isocratic conditions of 30% A and 70% B for 3 minutes at a flow rate of 0.9 ml/min. The column was flushed with 95%B for 2 minutes and re-equilibrated to initial conditions over 3 min.

UGT1A4 Quantitative Real-time-PCR

UGT1A4 RNA was quantified in the liver samples and expression levels were normalized for r18S content [29]. Briefly, reverse transcription reaction was performed using 200 units of Superscript II (Invitrogen, Burlington, Ontario, Canada) with 1 μg RNA, and 7.5 ng random hexamers (Roche, Laval, Québec, Canada) at 42°C for 50 minutes. UGT1A4 forward and reverse primer sequences for real-time PCR were 5'-CCT GTC CTA CAT TTG CCA TA-3' and 5'- TCA AAT TCC TGA GAT AGT GGC TTC CC-3', respectively. For each reaction, performed in triplicate, 50ng of cDNA was amplified in a 20μL total reaction volume, containing 10 μL SyBr Green PCR Mix (Applied Biosystems, Warrington, UK), 2 μL each primer (200 nM), and 6 μL of cDNA. Quantification was performed with an ABI Prism 7000 SDS v1.1 apparatus (Applied Biosystems, Warrington, UK). Amplification conditions were as follow: 10 minutes at 95ºC followed by 40 cycles of 15 seconds at 95ºC and 60 seconds at 60 ºC.

Statistical analyses

All statistical analyses were performed with the JMP software v4.0.2 (SAS Institute, Cary, NC). Normality of distribution was assessed using the Shapiro-Wilk W test. Logarithmic or Box Cox Y transformation was applied to values for which distribution was not normal. *P*-values of less than 0.05 for ANOVA or pairwise comparisons were considered statistically significant.

RESULTS

Sequencing of the UGT1A4 gene

Sequencing of the *UGT1A4* exonic region in Caucasian and African-American samples as well as in a set of liver samples, allowed the identification of 14 nucleotide changes in the first exon, including 10 resulting in amino acid change ($Arg^{3}Thr$, $Arg^{11}Trp$, $Pro^{24}Thr$, $Leu^{48}Val$, $Glu^{50}Asp$, $His^{56}Gln$, $His^{68}Tyr$, $Ile^{176}Phe$, $Ser^{250}Asn$ and $Ile^{276}Leu$) (Fig. 1A). In the 100 Caucasian individuals, 13 intronic and 39 promoter polymorphisms were found. Linkage disequilibrium (LD) analyses revealed a strong linkage between most promoter SNPs and also between intronic SNPs, resulting in two block structures, which were also in relatively elevated linkage (67%) (Fig. 2). Haplotypes were inferred in the cohort of Caucasian individuals (n=100) and revealed the existence nine different haplotypes of variable frequencies (Fig. 3). A series of variations in the promoter region (haplotypes *3a, *3c and *3d) were found to be in complete LD with the previously reported $L^{48}V$ coding region variant, with an allelic frequency of 6%. These variations were also frequently associated with a series of intronic variations (haplotypes *3c and *3d).

Computational analysis of *UGT1A4* promoter variations

To better assess if these variants could potentially modulate the transcription of the *UGT1A4* gene, a transcription factor binding site localization analysis was performed with the MatInspector software (Genomatix, Ann Arbor, MI). A region comprising the first 500 bp of the *UGT1A4* promoter was found to be associated with deletions or

additions of putative HNF-1 and OCT-1 binding sites in accordance with genotype status at positions -163, -219, -419 and -457 (Fig. 1B). While the variant at positions -419 was associated the addition of an OCT-1 binding site, the variant at position -457 was associated with the simultaneous deletion of an OCT-1 and the addition of an HNF-1 binding sites. Nucleotide changes were all found to be located in the core sequences of putative binding domains. Consequently, this variant haplotype is predicted to result in the putative displacement of one OCT-1 and addition of one HNF-1 binding sites.

UGT1A4 promoter variants and gene expression

Transcriptional activity modulation by the *UGT1A4* promoter variations was assessed by transient transfections in ACHN (kidney), Caco-2 (colon) and HepG2 (liver) cell lines. Although the basal activity was markedly reduced with the -163A variant in all cell lines, basal activity of the complete variant haplotype (-163A, -219T, -419A, -457T) did not differ significantly from the reference promoter (Fig. 4). The addition of HNF-1 and OCT-1 putative sites increased the luciferase activity in HepG-2 cells while no notable increase was detected in the other two cell lines. The infrequent -204G>A polymorphism, which demonstrated no evidence of LD with other proximal variations, enhanced the positive action of co-expressed HNF-1 on UGT1A4 promoter activity, and this effect was further increased by co-expression of OCT-1 in Caco-2 and HepG2 cells. However, no significant differences were observed in luciferase activity between the reference and variant promoter (-163A, -219T, -419A, -457T) constructs with the addition of either or both the HNF-1 and OCT-1 transcription factors in two of the three cell lines (Fig. 5). These results suggest that those promoter variants, using a proximal

construction around 500 bp of the *UGT1A4* promoter, do not substantially modify gene expression.

UGT1A4 coding variants modulate *in vitro* glucuronide formation

Several *UGT1A4* coding variants have been previously shown to alter *in vitro* glucuronidation. To confirm and extend upon these observations, we performed enzymatic assays and included the less frequent coding variations. For these assays, tamoxifen and Z-4-hydroxytamoxifen were tested (Table 2). Kinetics for tamoxifen fitted the Michaelis-Menten model. Several variants presented an altered K_m and/or V_{max} compared to the UGT1A4*1 reference protein. This resulted in a 105% increased V_{max}/K_m for UGT1A4*3 (Val^{48}), and a V_{max}/K_m for Asp^{50}, Gln^{56}, Phe^{176}, Asn^{250}, Leu^{276} 11-38% of the reference UGT1A4*1 protein. While relative V_{max} for z-4-OH-tamoxifen was reduced for all tested variants but UGT1A4*4 (Trp^{11}), K_m was increased 2-10 fold for variants Phe^{176}, Asn^{250}, Leu^{276}, resulting in a significantly reduced V_{max}/K_m for variants Asp^{50}, Gln^{56}, Phe^{176}, Asn^{250}, Leu^{276}. Variants Phe^{176} and Leu^{276}, when present together with Thr^{24} or Val^{48}, respectively, also resulted in markedly reduced the V_{max}/K_m for these two substrates.

Correlation between the presence of genetic variations, UGT1A4 mRNA, and glucuronidation activity in a human liver bank

In order to study the potential influence of identified *UGT1A4* variants, *UGT1A4* RNA expression and formation of TFP-glucuronide, as well as glucuronide 1 of N-OH-PhIP (uncharacterized at this time), were correlated with *UGT1A4* genotypes (-2497A>G,

-2170C>T, -1663C>T, -1484A>G, -1180G>A, Arg^{3}Thr, Arg^{11}Trp, Thr^{14}, Leu^{18}Phe, Pro^{24}Thr, Leu^{48}Val) in a set of liver samples. Firstly, *UGT1A4* RNA content in liver samples demonstrated a wide distribution (20±26 arbitrary units; range 1-117). Although RNA content correlated weakly with the extent of TFP-gluruconide formation (r2=0.301; p=0.047), it did not correlate with N-OH-PhIP glucuronide 1. A lower N-OH-PhIP glucuronide formation was observed for the -204A carriers (n=3; 13.2±7.6 *vs* 7.2±0.7nmoles/mg/min for -204G vs A carriers, respectively; p = 0.0132) (Fig. 6). Interestingly, a carrier of a silent variation at codon 14 (42G) had a significantly lower TFP glucuronidation (0.52±0.25 vs 0.03 for 42A *vs* G carriers, respectively; p=0.0005 rank for G carrier = 1). No significant correlation could be established between the other promoter and coding region variations and *UGT1A4* RNA expression or hepatic glucuronidation activities.

DISCUSSION

UGT1A4 is the one of the major enzymes responsible for the N-glucuronidation of various compounds, including therapeutic drugs [5-7]. In previous studies, UGT1A4 was demonstrated to have the capacity to conjugate antihistamines (cyproheptadine), tricyclic antidepressants (imipramine) and of numerous antipsychotics (clozapine, trifluoperazine) [10,15,30]. UGT1A4 was also found to generate tamoxifen and 4-OH-tamoxifen glucuronides [11], as well as one of the glucuronides of N-OH-PhIP, a dietary carcinogen found in cooked meat [9,10]. For a number of these compounds, metabolic variations were predicted to originate from individual genetic profiles of UGT enzymes. While a number of coding region polymorphisms, including the $P^{24}T$ and $L^{48}V$ variants, have been described, the 5'-untranslated region of the UGT1A4 gene has not been as extensively studied [18,31]. Although *UGT1A4* variations have previously been described in the Caucasian and Japanese population [15-17], to our knowledge, the present study is the most extensive to have been published so far.

Several coding variants in the *UGT1A4* gene significantly modify the activity of recombinant UGT1A4 for tamoxifen and Z-4-hydroxytamoxifen. The frequencies of non-synonymous variants established in this study are similar to those reported previously: 8% for the T^{24} variant and of 9-17% for the V^{48} variant [15,30]. We also demonstrated a high density of promoter variations. However, according to the *in vitro* analysis of the first 500 base pairs of its promoter region, predicted to be involved in gene regulation according to transcription factors binding sites analyses, no polymorphisms are significantly associated with a modulation of transcriptional activity, or trifluoperazine glucuronidation activity in liver microsomes.

Of all promoter variants found herein, haplotypes *3a, *3c and *3d were of particular interest due to their tight LD with the V^{48} coding variation. Transient transfections in various cell lines derived from human liver, kidney or colon, did not reveal markedly modified transcriptional activity. The HNF-1 and OCT-1 transcription factors are known regulators of *UGT* genes. HNF-1 was found to regulate several UGT1A and 2B genes, including UGT1A4 [32]. In turn, OCT-1 was previously reported to bind to HNF-1, consequently enhancing its capacity to activate the *UGT2B7* promoter [33]. The increased activity observed in the hepatic cell line HepG2 upon co-transfection with these transcription factors confirms their likely role in UGT1A4 regulation. In a previous study, the -163T variation was found to be associated with significantly reduced transcriptional activity upon transient transfection [19]. Although a similar observation was done in our study, the complete haplotype (-163T, -219A, 419T and -463A) did not show such a repression, despite its possible association with the addition and deletion of HNF-1 and OCT-1 putative binding sites. Since several other SNPs have also been found in the region beyond -1000 bp, additional studies will be required to carefully assess the contribution of the distal promoter in the transcriptional regulation of the *UGT1A4* gene and the potential influence of variable amino acids.

Kinetic analysis with tamoxifen and 4-hydroxytamoxifen have been previously published [11,14], while only the effect of variants $P^{24}T$ and $L^{48}V$ had been assessed for these substrates. The apparent K_m derived in this study were in a range similar to those observed by Kaku *et al.* [11], but higher than those noted by Sun *et al.* [14]. No significant effect was evidenced for the T^{24} allozyme compared to the reference UGT1A4.1 protein, but an increased glucuronidation activity for the V^{48} variant was

reported against tamoxifen and 4-OH-tamoxifen [14]. In the present study, a significantly increased activity was observed only for tamoxifen. We also identified additional novel although rare variations in the *UGT1A4* gene, including Asp^{50}, Gln^{56}, Phe^{176}, Asn^{250}, and Leu^{276}, all of which are likely to be functionally significant since they are associated with reduced UGT1A4 activity with one or both substrates.

Despite the fact that several variants are predicted to modulate glucuronidation based on *in vitro* functional date, this could not be demonstrated by correlative studies in a set of 52 human liver microsomes. The haplotype comprising the V^{48} coding region variant in addition to the four linked nucleotide changes at positions -163T, -219A, -419T and -457A, was not associated with significantly different N-OH-PhIP or TFP glucuronidation activities. Only the -204 variant appeared to change N-OH-PhIP glucuronidation, although this variation was found in only 3 liver samples. The T^{24} variant was only found in four liver samples and this limited our capacity to determine functional significance. Additional studies are required in order to assess the *in vivo* impact of genetic variations in *UGT1A4*.

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Table 1 Primer sequences

Location in *UGT1A4*	Sense	Primer Sequence (5'-3')	Annealing temperature °C
Promoter			
-5152 to -4024 bp	**F***	TGTTTCTGCTCCTTATGCAAGCCT	57
	R*	TGGAATAGCCACATTTCAAGTGCTC	
-4231 to -3176 bp	**F**	TCTAGTGGGCATGTTAAACACCAG	65
	R	TTATCCAGGTGTGATGGTGCACA	
	F*	CCACTAAAATTAATGTGAATA	
	R*	TGGAATAGCCACATTTCAAGTGCTC	
	F*	AGATGGAGTCTTGCTCTGTCACCCA	
	R*	AGGCGCATGGCACCACATCCAGC	
	F*	AGATGGAGTTTCATCATATTGGCCA	
	R*	GGTGCATGTAGGTTTGAGGTTTG	
	R*	GCAACATGCTGAGACTCCATCT	
-3297 to -1959 bp	**F**	CCTTTGGGAGACCGAGGG	58
	R	GCTACCACATATGCTGATGGC	
	F*	GCAGCATCTTCAAGTCTCTAAT	
	R*	ATAAAGTCTCTTCCACCTG	
-2080 to -742 bp	**F**	GATTGGGAGAGGGGAGCTAGAT	55
	R	GTGGGTCCTTGCTAGGGTTGT	

	F*	GCTGAGAATCCCTTTCTAGCA	
	R*	ATATGCCTCCCTTGAGCTGGG	
-1013 to +22bp	**F**	TTATGCAGCCCGTTCTGTTCTGGA	56
	R	GAGATGGCCAGAGGACTCCAGGTTC	
	F*	GACTTGGAGAAAAGCCTGG	
Exon 1			
-108 to +701bp	**F**	CTGATTTGCTAGGTGGCTCAA	56
	R	CCCTTATGCAAGTCTTGCC	
	F*	CTGACAGCCTATGCTGTTC	
	R*	GTATCTTTGGCCCTTCATAGGTG	
+229 to IV1S+159bp	**F**	CTGACAGCCTATGCTGTTC	56
	R	GTTTTCAGTGGTCACTGAGAG	
	F*	TCCTTCCTCCTATATTCCTAAG	
	R*	CCCTTATGCAAGTCTTGCC	
Intron 1			
+687 to IV1S+1017bp	**F**	TTATGCAAGTCTTGCCTCTGAGC	61
	R	AAATCCATTAAGGGAGCCATCC	
	F*	TATGCATCCGTGTGGCTGTTCCGA	
	F*	CTCAGGTGAAGCTGATCATATCA	
IV1S+934bp to IV1S+1988bp	**F**	TCATTGAAATAGTACTCTGGGATG	55
	R	GGTCATGTAAGGGTTAATCCAAT	
	F*	TTCTTGAGACTGAGCCTCGTTCTG	

	R*	CATGTGCCACTGCTCCTGGCAATT

PCR primers are indicated in bold. *Indicates primers used for sequencing.

Reference sequence is AF297093. Position is relative to the first nucleotide of each primer. F, forward; R, reverse.

Table 2 *UGT1A4* coding variants and tamoxifen and trans-4-hydroxytamoxifen glucuronidation activities.

	Apparent K_m (μM)	K_i (μM)	n	Relative V_{max} (pmol/min/mg protein)	V_{max} / K_m (μl/min/mg)
Tamoxifen					
1A4*1	17.7 ± 2.0			565 ± 35	32.3 ± 5.7
1A4*2	22.2 ± 1.1			568 ± 62	28.2 ± 5.3
1A4*3	6.7 ± 0.7 *			440 ± 23	66.2 ± 3.6
1A4*4	16.8 ± 3.4			416 ± 12 *	25.4 ± 5.8
1A4*9	14.2 ± 2.5			507 ± 58	36.5 ± 10.4
1A4*10	17.0 ± 2.3			200 ± 7 *	11.8 ± 1.2
1A4*11	70.7 ± 18.4			285 ± 1 *	4.2 ± 1.1
1A4*12	11.7 ± 0.8			419 ± 115	36.3 ± 12.3
1A4*13	9.7 ± 0.5 *			117 ± 3 *	12.1 ± 0.9
1A4*14	14.6 ± 5.1			262 ± 1 *	19.1 ± 6.7
1A4*15	51.9 ± 2.0 *			177 ± 16 *	3.4 ± 0.2
1A4*16	7.3 ± 0.2 *			318 ± 17 *	43.3 ± 3.5
1A4*17	43.4 ± 13.2			93 ± 16 *	2.3 ± 1.1
1A4*18	7.9 ± 2.4 *			31 ± 3 *	4.1 ± 1.6
z-4-OH-tamoxifen					
1A4*1	15.4 ± 1.5	261 ± 24	±	2193 ± 181	143.4 ± 25.4
1A4*2	15.2 ± 1.1	242 ± 46	±	1151 ± 162 *	80.0 ± 11.3
1A4*3	10.4 ± 1.1	217 ± 17	±	1528 ± 77 *	147.8 ± 23.3
1A4*4	12.9 ± 1.2	214 ± 14	±	1560 ± 241	122.1 ± 30.0
1A4*9	15.5 ± 1.6	314 ± 27	±	1025 ± 20 *	66.6 ± 8.0

1A4*10	18.3	±	0.6		252	±	16			±		434	±	33	*	23.7	±	2.6
1A4*11	20.6	±	1.4		222	±	0			±		197	±	12	*	9.6	±	0.1
1A4*12	16.6	±	0.3		319	±	46			±		1098	±	45	*	66.0	±	3.7
1A4*13	99.1	±	9.9	*				1.70	±	0.13		357	±	28	*	3.6	±	0.6
1A4*14	160.5	±	16.6	*				1.09	±	0.07		289	±	24	*	1.8	±	0.3
1A4*15	24.3	±	1.3	*	247	±	14			±		109	±	12	*	4.5	±	0.3
1A4*16	16.3	±	1.1		357	±	35			±		1348	±	48	*	83.1	±	8.7
1A4*17	31.5	±	4.2	*	256	±	1			±		351	±	31	*	11.2	±	0.5
1A4*18	92.1	±	0.1	*				1.69	±	0.04		22	±	5	*	0.2	±	0.1

Vmax was adjusted for relative expression of each variant isoform: UGT1A4*1=1 (arbitrarily set at 1), UGT1A4*2=0.07, UGT1A4*3=0.45, UGT1A4*4=0.66, UGT1A4*9 (T3)=0.31, UGT1A4*10 (D50)=0.86, UGT1A4*11 (Q56)=0.47, UGT1A4*12 (Y68)=0.21, UGT1A4*13 (F176)=1.09, UGT1A4*14 (N250)=0.50, UGT1A4*15 (L276)=0.51, UGT1A4*16 (V48Y68)=0.94, UGT1A4*17 (T24L276)=0.44, UGT1A4*18 (V48F176)=0.16.

For 4-OH-tamoxifen, UGT1A4*13 (F176), UGT1A4*14 (N250) and UGT1A4*18 (V48F176) were characterized by a Hill's profile.

LEGENDS FOR FIGURES

Fig. 1 (A) Sequencing of the *UGT1A4* gene allowed the identification of 66 polymorphisms. (B) *In silico* analyses revealed that four polymorphisms located in the first 500 bp were associated with deletions or additions of putative HNF-1 and OCT-1 transcription factors binding sites.

Fig. 2 Pairwise LD and bloc structure of *UGT1A4* polymorphisms.

Fig. 3 *UGT1A4* haplotypes and their frequencies in Caucasian individuals (n=100). Variants at codons 24 and 48 were already reported.

Fig. 4 Transient transfections of reference and variant *UGT1A4* promoter constructs in diverse human cell lines.

Fig. 5 Cotransfections of reference and variant *UGT1A4* promoter constructs (500 bp) with HNF-1 and/or OCT-1 in ACHN, Caco-2 and HepG2 cell lines do not result in marked modification of expression. * $p \leq 0.05$

Fig. 6 Relationship between N-OH-PhIP glucuronidation rates and the *UGT1A4* -204 variation.

Figure 1

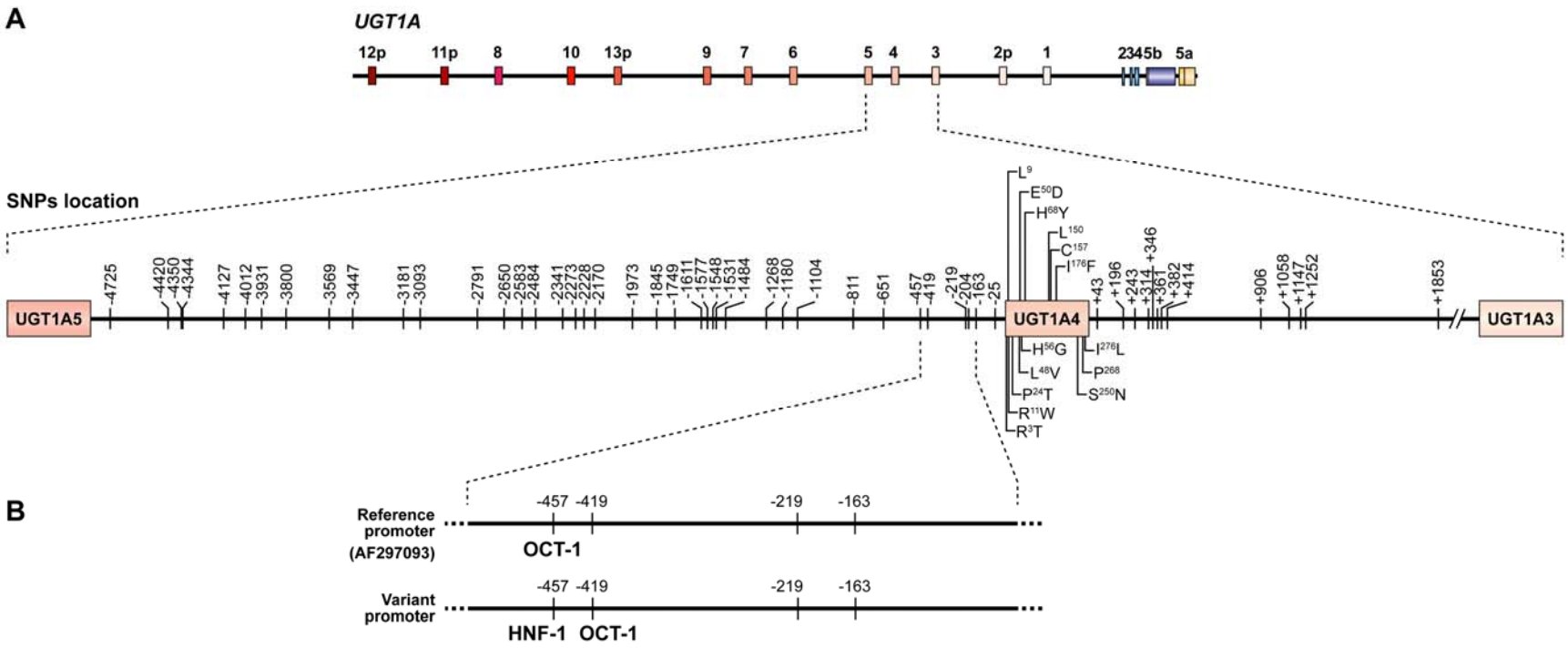

A
UGT1A
12p
11p
8
10
13p
9
7
6
5
4
3
2p
1
2345b
5a
SNPs location
UGT1A5
UGT1A4
UGT1A3
B
Reference promoter (AF297093)
Variant promoter
OCT-1
HNF-1
OCT-1

Figure 2

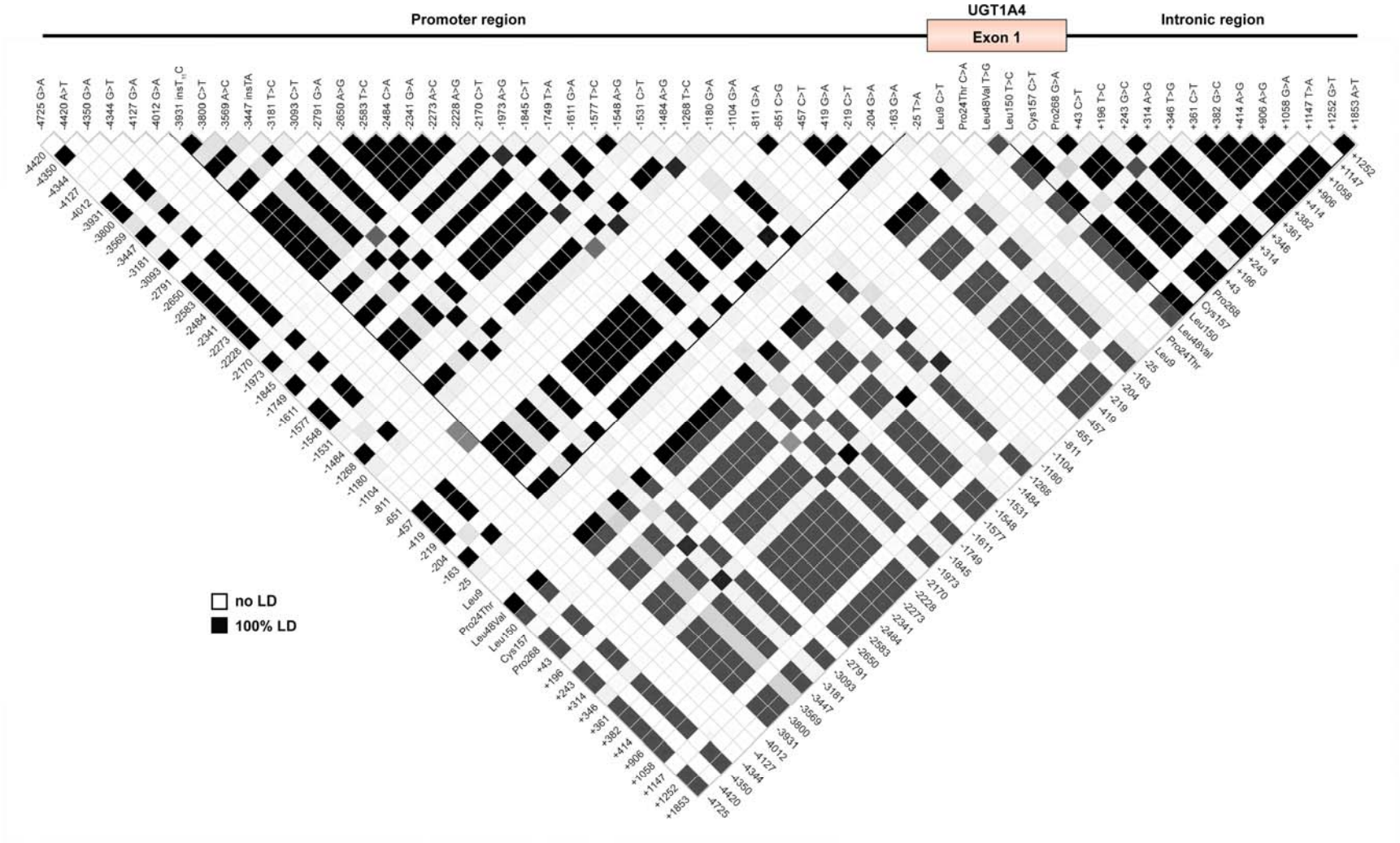
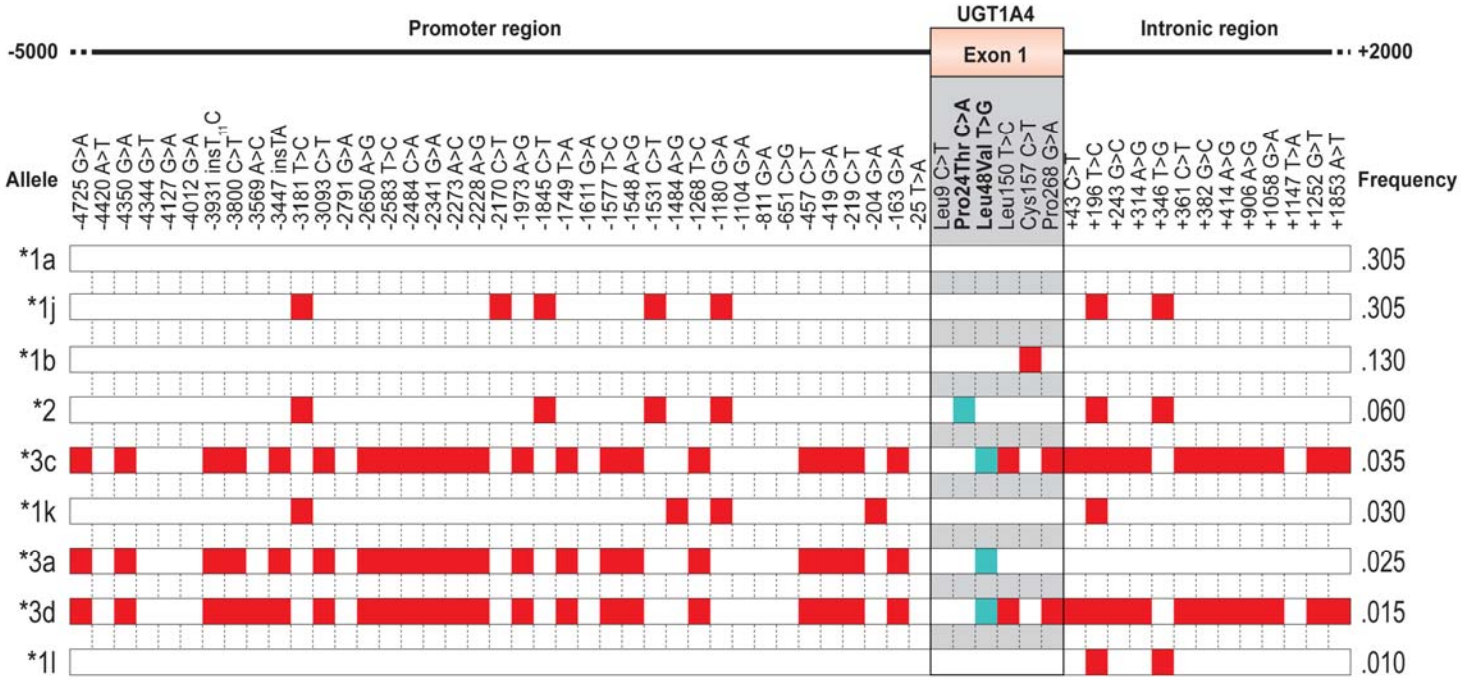


Figure 3


UGT1A4
Promoter region
Exon 1
Intronic region
-5000
+2000
Allele
Frequency
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-4420 A>T
-4350 G>A
-4344 G>T
-4127 G>A
-4012 G>A
-3931 insT11C
-3800 C>T
-3569 A>C
-3447 insTA
-3181 T>C
-3093 C>T
-2791 G>A
-2650 A>G
-2583 T>C
-2484 C>A
-2341 G>A
-2273 A>C
-2228 A>G
-2170 C>T
-1973 A>G
-1845 C>T
-1749 T>A
-1611 G>A
-1577 T>C
-1548 A>G
-1531 C>T
-1484 A>G
-1268 T>C
-1180 G>A
-1104 G>A
-811 G>A
-651 C>G
-457 C>T
-419 G>A
-219 C>T
-204 G>A
-163 G>A
-25 T>A
Leu9 C>T
Pro24Thr C>A
Leu48Val T>G
Leu150 T>C
Cys157 C>T
Pro268 G>A
+43 C>T
+196 T>C
+243 G>C
+314 A>G
+346 T>G
+361 C>T
+382 G>C
+414 A>G
+906 A>G
+1058 G>A
+1147 T>A
+1252 G>T
+1853 A>T
*1a .305
*1j .305
*1b .130
*2 .060
*3c .035
*1k .030
*3a .025
*3d .015
*1l .010

Figure 4

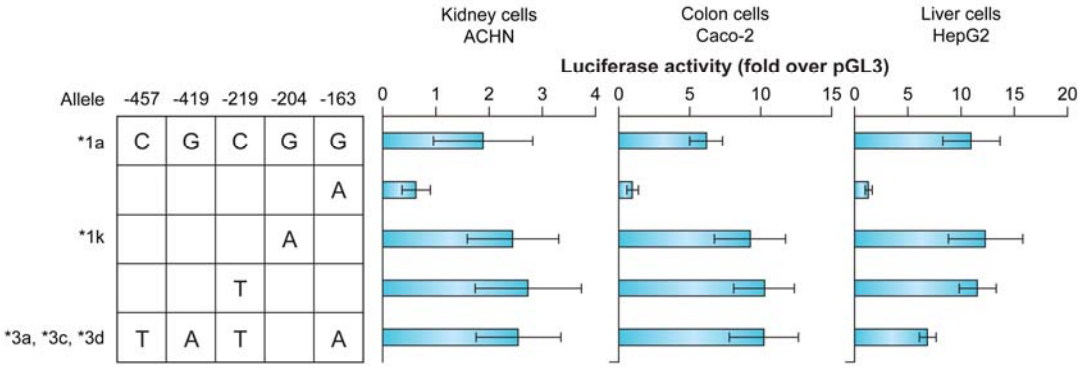


Figure 5

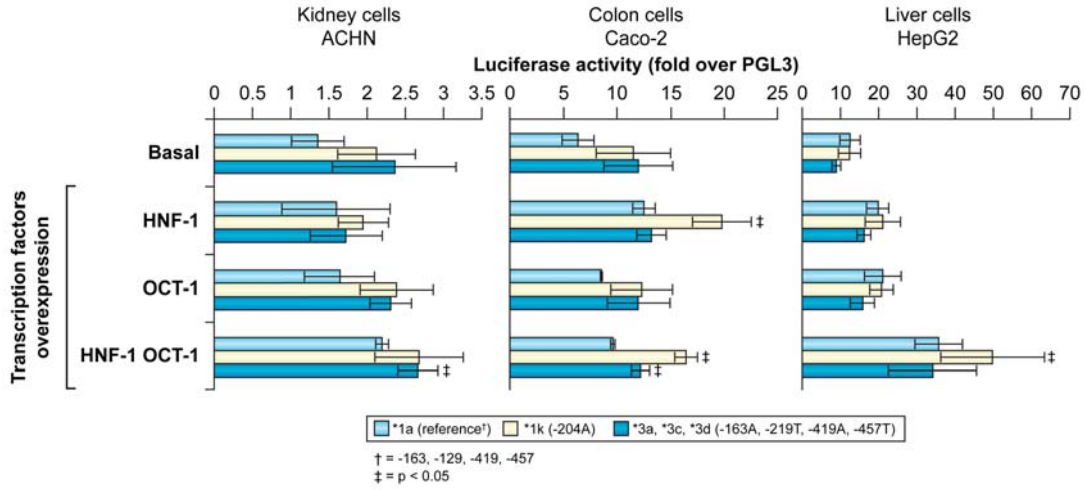
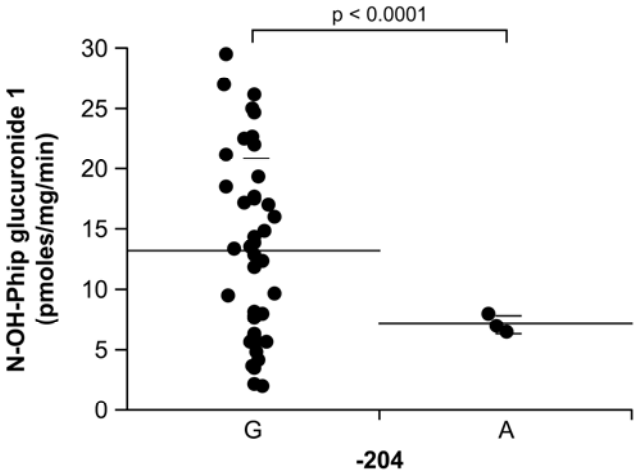


Figure 6


p < 0.0001
N-OH-Phip glucuronide 1
(pmoles/mg/min)
30
25
20
15
10
5
0
G
A
-204