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IMPORTANCE OF UDP-GLUCURONOSYLTRANSFERASE 1A10 (UGT1A10) IN THE DETOXIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS: DECREASED GLUCURONIDATIVE ACTIVITY OF THE UGT1A10^{139LYS} ISOFORM

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Running title: Functional analysis of UGT1A10^{139Lys} polymorphism

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Abbreviations: UGT, UDP-glucuronosyltransferase; PAH, polycyclic aromatic hydrocarbons; BaP, benzo(a)pyrene; 3-OH-BaP, 3-hydroxy-benzo(a)pyrene; 7-OH-BaP, 7-hydroxy-benzo(a)pyrene; 9-OH-BaP, 9-hydroxy-benzo(a)pyrene; BPD, BaP-*trans*-7,8-dihydrodiol; 1-OH-pyrene, 1-hydroxypyrene; UDPGA, UDP-glucuronic acid; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; TLC, thin layer chromatography.

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ABSTRACT

UGT1A10 is an extra-hepatic enzyme expressed in aerodigestive tract tissues that exhibits significant glucuronidation activity against the important procarcinogenic benzo(a)pyrene (BaP) metabolite, BaP-7,8-dihydrodiol (BPD), and the UGT1A10 codon 139 (Glu>Lys) polymorphism was previously implicated in risk for orolaryngeal cancer (Elahi et al, *Cancer* **15**:872-880; 2003). To better assess the potential role of UGT1A10 in risk for tobacco-related cancers, the glucuronidation activity of UGT1A10 was compared to that of other known UGT enzymes against selected polycyclic aromatic hydrocarbons and the effects of the codon 139 polymorphism on UGT1A10 function was examined *in vitro*. UGT1A10 exhibited considerably more glucuronidation activity as determined by V_{max}/K_m against 3-hydroxy (OH)-BaP, 7-OH-BaP, 9-OH-BaP and 1-OH-pyrene than any other UGT1A family member. Although a kinetic comparison utilizing V_{max} could not be performed against family 2B UGTs, UGT1A10 exhibited a 1.7-254-fold lower K_m than active family 2B UGTs against 3-OH-BaP, 7-OH-BaP and 1-OH-pyrene. A significantly (p<0.01) higher V_{max}/K_m was observed for homogenates from wild-type UGT1A10^{139Glu}-over-expressing cells against all four BaP metabolites tested (3-OH-BaP, 7-OH-BaP, 9-OH-BaP and BPD). A similarly significant (p<0.05) increase in V_{max}/K_m was observed for homogenates from wild-type UGT1A10^{139Glu}-over-expressing cells against 1-OH-pyrene. Significant differences in K_m were observed for homogenates from wild-type UGT1A10^{139Glu}-over-expressing cells against 1-OH-pyrene (p<0.05) and 3-OH-BaP (p<0.01). RT-PCR of total lung RNA demonstrated low levels

of UGT1A10 expression in human lung tissue. Together, these studies implicate UGT1A10 as an important detoxifier of PAHs in humans and that the UGT1A10 codon 139 polymorphism may be an important determinant in risk for tobacco-related cancers.

Introduction

The UGT superfamily of enzymes catalyze the glucuronidation of a variety of endogenous compounds such as bilirubin and steroid hormones, as well as xenobiotics such as drugs and environmental carcinogens (Tephly and Burchell, 1990; Owens and Ritter, 1995; Gueraud and Paris, 1998; Ren et al., 2000). Based upon structural as well as sequence homology, UGTs are classified into several families and subfamilies (Jin et al., 1993). UGT family 2B members are derived from independent genes, whereas the entire UGT1A family is derived from a single gene locus in chromosome 2. This loci codes for nine functional proteins that differ only in their amino-terminus as a result of alternate splicing of independent exon 1 regions to a shared carboxy-terminus encoded by exons 2-5 (Beaulieu et al., 1997). Several family 1A UGTs have been implicated in the conjugation and detoxification of tobacco carcinogen metabolites including the tobacco-specific nitrosamine, NNK (Ren et al, 2000; Wiener et al, 2004a) and PAHs like BaP (Ciotti et al., 1997; Levesque et al., 1997; Beaulieu et al., 1998; Bélanger et al., 1998; Carrier et al., 2000). While most family 1A UGTs are expressed in the liver (Ciotti et al., 1997; Burchell and Hume, 1999; Levesque et al., 1999; Guillemette et al., 2000a), several UGTs are extra-hepatic (Tukey and Strassburg, 2000) and are expressed in several target tissues for tobacco-induced cancers including tissues in the aerodigestive tract (Zheng et al, 2002).

Polymorphisms have been previously identified for many of the UGT genes and several recent studies have examined their potential role in tobacco carcinogenesis and in risk for tobacco-induced cancers. In studies examining UGT family 1A variants, the

'TATA' box polymorphism in the promoter region of UGT1A1, commonly associated with Gilbert's Syndrome, is associated with reduced function in the UGT1A1 transcriptional promoter (Burchell and Hume, 1999) and is associated with decreased formation of the glucuronide conjugate of the important procarcinogenic BaP metabolite, BPD, in liver microsomes (Fang and Lazarus, 2004). UGT1A7-specific genetic variants are associated with reduced UGT1A7 metabolic function against BaP phenols (Guillemette et al., 2000a) and are strongly linked to increased risk for orolaryngeal (Zheng et al., 2001), pancreatic (Ockenga et al, 2003), and lung (Araki et al, 2005) cancer. Recent studies have demonstrated an association between liver microsomal *O*-glucuronide conjugate formation activity against NNAL, the major metabolite of NNK, and a Pro>Thr polymorphism at codon 24 of the UGT1A4 gene (Wiener et al., 2004b). Among the family of 2B polymorphic variants, recent studies have demonstrated an association between both the UGT2B7 codon 268 polymorphism as well as the UGT2B17 gene deletion polymorphism and the *O*-glucuronidation of NNAL in liver microsomes (Wiener et al., 2004b; Lazarus et al, 2005).

UGT1A10 is an extra-hepatic enzyme expressed in aerodigestive tract tissues (Zheng et al, 2002) that exhibits significant glucuronidation activity against BPD (Fang et al, 2002). Previous studies have demonstrated that polymorphism in codon 139 of the UGT1A10 gene, resulting in a non-conservative Glu>Lys amino acid change, was linked to altered risk for orolaryngeal cancer (Elahi et al., 2003). No such association was observed for the UGT1A10 codon 244 polymorphism, which results in a conservative amino acid change of Leu>Ile. To better assess the potential role of UGT1A10 in risk for tobacco-related cancers, the glucuronidation activity of UGT1A10

was compared to that of other known UGT enzymes against selected PAHs, UGT1A10 expression was examined in lung tissue, and the effects of the codon 139 polymorphism on UGT1A10 function was examined *in vitro*. Results are presented demonstrating that UGT1A10 appears to be the most highly active UGT against a number of tobacco carcinogen metabolites, that UGT1A10 is expressed in lung, and that the UGT1A10^{139Lys}-encoded variant exhibits reduced enzyme activity against all substrates tested as compared to the wild-type UGT1A10^{139Glu}-encoded isoform *in vitro*.

MATERIALS AND METHODS

Chemicals and materials. 3-OH-BaP, 7-OH-BaP, 9-OH-BaP, and BPD were obtained from the National Cancer Institute Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). UDPGA, D,L-2-lysophosphatidyl choline palmital C16:0, 1-OH-pyrene, 1-naphthol, 4-nitrophenol, and 4-methylumbelliferone were purchased from Sigma (St. Louis, MO). ^{14}C-UDPGA (specific activity: 300 mCi/mmol) was obtained from PerkinElmer (Wellesley, MA). Dulbecco's Modified Eagle's Medium was obtained from Mediatech (Herndon, VA) and both fetal bovine serum and Geneticin (G418) were purchased from GIBCO (Carlsbad, CA). Taq DNA polymerase (HotMaster) was purchased from PerkinElmer Biosystems (Foster City, CA); M-MULV reverse transcriptase and the pcDNA3.1/V5-His-TOPO mammalian expression vector were obtained from Invitrogen (Carlsbad, CA); the human UGT1A western blotting kit that includes the anti-UGT1A polyclonal antibody was purchased from Gentest (Woburn, MA) while the anti-β-actin monoclonal antibody (1:5000 dilution) was provided by Sigma.

The human embryonic kidney HK293 cell line was purchased from ATCC (Rockville, MD) while the human oral squamous cell carcinoma MSK1483 cell line was kindly provided by Peter Sacks (New York University, New York). Cell lines stably over-expressing UGT1A4, UGT1A6, UGT1A8, UGT2B4 and UGT2B7 were previously-described (Ren et al, 2000; Wiener et al, 2004a). Baculosomes (Gentest) were utilized for screening UGT1A1, UGT1A3, UGT1A7, UGT1A9, UGT2B15 and UGT2B17 glucuronidation activities.

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UGT1A10 cloning and RT-PCR of lung tissue. The amplification of UGT1A10 cDNA was performed after an initial reverse transcriptase reaction using 3 μg of total RNA from the MSK1483 cell line (shown previously to express UGT1A10; unpublished data), 2.5 μM of oligo(dT) primer, and 200 units of reverse transcriptase in a 50 min incubation at 42^{o}C. PCR amplification was subsequently performed using 2 μl of the reverse transcriptase reaction in a 50 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of deoxynucleotide triphosphates, 20 pmole of both sense (UGT1A10S1, 5'-TCCGCCTACTGTATCATAGCAG-3', corresponding to nt –61 to –40 relative to the UGT1A10 translation start site; GenBank accession number BC020971), and antisense (UGT1A10AS1, 5'-TTTTACCTTATTTCCCACCC-3', corresponding to nt +6 to +25 relative to the UGT1A10 translation stop codon) primers, and 2.5 units of Pfx DNA polymerase. Incubations were performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) as follows: 1 cycle of 94°C for 2 min, 41 cycles of 94°C for 30 sec, 55° C for 30 sec, and 72°C for 2 min, followed by a final cycle of 7 min at 72°C. The PCR product (1679 bp) was purified after electrophoresis in 1.5% agarose using the QIAEX® II gel extraction kit (Qiagen, Valencia, CA) and subsequently sub-cloned into the pcDNA3.1/V5-His-TOPO mammalian expression vector using standard methodologies. Confirmation of insert orientation was performed by restriction enzyme digestion and the UGT1A10 sequence were confirmed by dideoxy sequencing of the entire PCR-amplified UGT1A10 cDNA product (performed at the Molecular Biology Core Facility at Penn

State University College of Medicine), using two vector primers (T7 and BGH, Integrated DNA Technologies, Coralville, IA) and one internal sense primer (UGT1A10S2; 5'-CCTCTTTCCTATGTCCCCAATGA-3', corresponding to nucleotides +556 to +578 relative to the UGT1A10 translation start site in the UGT1A10 cDNA by comparison to the UGT1A10 cDNA sequence described in GenBank (accession # BC020971).

RT-PCR from normal human lung tissue was performed as described above except that total RNA from histologically normal human lung tissue specimens, obtained from the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center, was used as template. The primers used to amplify exon 1 of UGT1A10 were described previously (Zheng et al, 2002). All protocols involving the analysis of tissue specimens were approved by the institutional review board at Penn State College of Medicine and in accordance with assurances filed with and approved by the United States Department of Health and Human Services.

Site-directed mutagenesis, generation of cell lines and cell homogenate preparation. The UGT1A10^{139Lys} variant was generated by PCR amplification of the pcDNA3.1/V5-His-TOPO vector containing the wild-type UGT1A10 sequence using site-directed mutagenesis primers specific for the polymorphic site. The primers used to generate this variant were UGT1A10-m139F (5'-GTAGAATACTTAAAG**A**AGAGTTCTT TTGATGCAGTGTTTCTGG-3') and UGT1A10-m139R (5'-CCAGAAACACTGCATCAA AAGAACTCT**T**CTTTAAGTATTCTAC-3'), corresponding to bases +400-442 relative to the UGT1A10 translation start site, with the polymorphic base in bold for both primers.

PCR was performed using 10 units of Pfx polymerase, 1X Pfx buffer, 2X enhancer solution (Invitrogen), 100-500 ng template, 1 mM MgSO4, 300 μM dNTPs, and 0.6-2.4 μM of each primer. The products were amplified in a BioRad MyCycler (Hercules, CA) with an initial denaturation of 95°C for 2 min, followed by 25 cycles of 95°C for 30 sec, 59-61°C for 30 sec, and 68°C for 18 min. Following amplification, 20 units of the DpnI restriction enzyme was added to each reaction and incubated for 1.5 h at 37°C to specifically digest the wild-type template DNA. The non-digested, PCR-amplified plasmid (which has incorporated the polymorphism) was then transformed into competent DH5α *E. coli*, individual colonies were isolated, and subsequent plasmid DNA minipreps screened for the codon 139 variant by digestion with the Ear*I* restriction enzyme which specifically recognizes the UGT1A10^{139Glu} but not the UGT1A10^{139Lys} variant. UGT1A10 sequences were confirmed by dideoxy DNA sequencing analysis utilizing the same primers used to confirm the cloning of wild-type UGT1A10 as described above.

HK293 cell lines over-expressing wild-type or variant UGT1A10 were generated by stable transfection using the LipofectAMINE^{TM} Reagent (Invitrogen,Carlsbad, CA) procedure according to the manufacturer's protocol. Briefly, pcDNA3.1/V5-His-TOPO/UGT1A10 constructs were transfected into HK293 cells grown in 5% CO_2 to 80% confluence in Dulbecco's Modified Eagle's medium supplemented with 4.5 mM glucose, 10 mM HEPES, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. At 24 h post-transfection, cells were passaged and subsequently grown in geneticin (700 μg/mL medium) for the selection of geneticin-resistant cells, with

selection medium changed every 3 to 4 days. Cell homogenates were prepared by re-suspending pelleted cells in Tris-buffered saline (25 mM Tris base, 138 mM NaCl, 2.7 mM KCl; pH 7.4) and subjecting them to 3 rounds of freeze-thaw prior to gentle homogenization. Cell homogenates (5 - 30 mg homogenate protein/mL) were stored at -70°C in 100 μL aliquots. Total cell homogenate protein concentrations were determined using the BCA assay from Pierce Biotechnology (Rockford, IL) after protein extraction using standard protocols.

Western blot analysis. Levels of UGT1A protein in UGT-over-expressing cell lines were measured by Western blot analysis using the anti-UGT1A antibody (1:5000 dilution as per the manufacturer's instructions), while β-actin protein levels were assayed using a 1:5000 dilution of the monoclonal anti-β-actin antibody. UGT1A protein was detected by chemiluminescence using the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Inc., Rockford, IL). Secondary antibodies supplied with the Dura ECL kit (anti-rabbit and anti-mouse) were used at 1:3000. UGT1A protein levels were quantified against a known amount of human UGT1A protein (100 ng, supplied in the Western blotting kit provided by Gentest) by densitometric analysis of X-ray film exposures (5 sec – 2 min exposures) of Western blots using a GS-800 densitometer with Quantity One software (Bio-Rad, Hercules, CA). Quantification was made relative to the levels of β-actin observed in each lane (also quantified by densitometric analysis of Western blots as described above). X-ray film bands were always below densitometer saturation levels as indicated by the

densitometer software. Relative UGT1A protein levels are reported as the mean of three independent Western blot experiments, with Western blot analysis performed using the same UGT1A-containing cell homogenates used for activity assays.

Glucuronidation Assays. The rate of glucuronidation by cell homogenates was determined essentially as previously described (Fang et al., 2002; Wiener et al., 2004b). Cell homogenate protein (0.10-3.0 mg) was incubated (100-250 µl final volume) in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, D,L-2-lysophosphatidyl choline palmital C16:0 (0.2 mg/mg protein), 4 mM UDPGA, and 0.025-5 mM aglycone at 37^{o}C for 1-2 h (as indicated in the text). ^{14}C-UDPGA (1µCi/100 µl reaction volume) was added to all assays except those incubations with PAHs as substrate. For glucuronidation rate determinations, aglycone concentrations, cell homogenate protein levels and incubation times for individual assays were chosen to maximize levels of detection within a linear range of uptake and were similar to established protocols (Fang et al., 2002; Wiener et al., 2004b). For kinetic analysis, incubations were performed using 0.2 mg (for analysis of 1-OH-pyrene, 3-OH-BaP, 7-OH-BaP, and 9-OH-BaP) or 1 mg (for analysis of BPD) UGT1A10-over-expressing cell protein homogenate, with equal amounts of protein homogenate assayed for the wild-type and variant UGT1A10-over-expressing cell lines in any given experiment. Aglycone concentrations ranged between 0.0025–1 mM, a range that encompassed the K_m for all metabolites tested. Reactions were terminated by the addition of an equal volume of 100% acetonitrile on ice. Glucuronidation assays were analyzed by high performance liquid chromatography (HPLC) with appropriate

controls as previously described (Ren et al, 2000; Fang et al, 2002; Wiener et al, 2004a). Experiments were always performed in triplicate as independent assays.

Statistical Analysis. The Student's t-test (2-sided) was used for comparing rates and kinetic values of glucuronide formation for the UGT1A10^{139Glu} and UGT1A10^{139Lys} isoforms against the different substrates examined in this study.

Results

Kinetic analysis of human UGT-isoforms activity against monohydroxylated PAHs. Several UGT family members have been shown to glucuronidate PAHs including metabolites of BaP, with UGT1A10 exhibiting higher glucuronidation activity against both the (-) and (+) isomers of BPD as compared to other UGT enzymes (Fang et al, 2002). To determine whether UGT1A10 exhibits a similarly high activity against other PAHs, the relative activities of individual UGT enzymes were screened against 1-OH-pyrene as well as three hydroxylated metabolites of BaP, 3-OH-BaP, 7-OH-BaP, and 9-OH-BaP. In screening assays using cell homogenate or baculosomal protein for individually over-expressed UGT enzymes, 1-OH-pyrene glucuronidation activity was detected for all of the UGTs tested except UGT1A4 and UGT2B4 (Table 1). The same pattern of activity was observed against all of the monohydroxylated BaP metabolites tested, except that homogenates from UGT1A6-over-expressing cells exhibited no detectable activity against any of these substrates. Cell homogenates from cell lines over-expressing UGT1A4 or UGT2B4 were active against other known aglycones (results not shown). For kinetic analysis, family 1A UGT activities were normalized based on UGT1A expression in the individual UGT-over-expressing cell homogenates or baculosomes based on Western blot analysis (Figure 1, panel A). A similar analysis for the UGT2Bs was not performed since an antibody that recognizes all UGT2B family members was not available. As shown in Table 1, UGT1A10 was considerably more active against all PAHs tested than any other UGT1A family member as determined by V_{max}/K_m (Table 1). UGT1A9 also

exhibited significant activity against all three BaP metabolites as determined by V_{max}/K_m, and UGT1A8 exhibited similar activity to UGT1A9 against 3-OH-BaP. UGT1A enzymes exhibiting significant activity as determined by V_{max}/K_m against 1-OH-pyrene in addition to UGT1A10 were UGT1A6 and UGT1A8. Although a comparison utilizing V_{max} could not be performed against family 2B UGTs, UGT1A10 exhibited a 1.7-254-fold lower apparent K_m than active family 2B UGTs against 3-OH-BaP, 7-OH-BaP and 1-OH-pyrene. Overall, the affinity of individual UGT enzymes as determined by apparent K_m analysis was 1A10 > 1A1 $\geq$ 1A9 > 1A7 > 1A8 > 2B17 > 1A3 > 2B15 > 2B7 for 3-OH-BaP, 1A10 > 1A3 $\geq$ 1A9 > 1A7 > 2B7 $\geq$ 1A8 > 1A1 > 2B15 > 2B17 for 7-OH-BaP, 1A7 $\geq$ 1A10 > 2B7 > 2B15 > 1A9 > 1A3 $\sim$ 1A1 $\geq$ 2B17 > 1A8 for 9-OH-BaP, and 1A10 > 1A9 $\geq$ 1A7 > 2B17 $\geq$ 1A1 $\geq$ 1A6 > 2B15 > 1A8 > 1A3 > 2B7 for 1-OH-pyrene.

Expression of UGT1A10 in human lung. Previous studies have demonstrated that UGT1A10 and UGT1A7 are the only UGTs expressed in all aerodigestive tract tissues examined, including floor of mouth, larynx, tonsil, esophagus, and tongue (Zheng et al, 2002). In the same studies, UGT1A10 was not detected in lung tissue by multiplex RT-PCR. Recent studies have suggested that UGT1A10 was expressed in an irinotecan-resistant lung adenocarcinoma cell line and capable of glucuronidating SN-38, the active metabolite of irinotecan (Oguri et al, 2004). To better assess whether UGT1A10 is in fact expressed in human lung, RT-PCR of UGT1A10 exon 1 alone or in a multiplex assay with β-actin was performed using total RNA extracted from normal human lung tissue. As shown for a representative lung RNA specimen (Figure 1B), UGT1A10 was expressed in normal human lung tissue, but only when assayed as a

single amplification with one primer set specific for UGT1A10 (lane 4); consistent with previously reported results (Zheng et al, 2002), UGT1A10 was not detected in human lung when multiplex RT-PCR was performed (lane 6). The amplified product was confirmed to be UGT1A10 by sequencing analysis (data not shown). Similar results were obtained in RT-PCR assays for multiple human lung specimens (data not shown). No UGT1A10 expression was detected using HK293 cell total RNA as template when assays were performed using UGT1A10 primers alone or when multiplexed with primers for β-actin (results not shown). This is the first demonstration of UGT1A10 expression in the human lung.

Analysis of UGT1A10^{139Lys} function using UGT1A10-over-expressing cell lines. To determine whether the Glu>Lys amino acid change at codon 139 of the UGT1A10 gene affects UGT1A10 enzyme activity, stable HK293 cell lines over-expressing either the UGT1A10^{139Glu} or UGT1A10^{139Lys} isoforms were created. The over-expressed UGT1A10 wild-type and polymorphic cDNAs were PCR-amplified and fully sequenced as described in the Materials and Methods. The UGT1A10 cDNA sequence of both cell lines matched with 100% identity to the UGT1A10 cDNA sequence described in GenBank (accession number BC030974) with the only difference between the cell lines being the polymorphic G>A transition at nt +415 in codon 139, resulting in a Glu>Lys amino acid change. Semi-quantitative Western blot analysis showed high levels of UGT1A10 protein in homogenates of both the UGT1A10^{139Glu}- and UGT1A10^{139Lys}-over-expressing HK293 cell lines (Figure 1C). The

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levels of UGT1A10 relative to β-actin (as an internal reference for expression) measured by densitometry revealed that the level of expression of the UGT1A10^{139Lys} polymorphic variant was 2.1-fold greater than the expression of the UGT1A10^{139Glu} wild-type isoform. No UGT1A expression was detected by Western blot analysis in the HK293 cell line.

As shown in a representative chromatogram (Figure 2), glucuronidation activity was clearly discernable for both the UGT1A10^{139Glu} and UGT1A10^{139Lys} cell lines against 4-nitrophenol (top and middle panels). A 4-nitrophenol-glucuronide peak (retention time = 12 min) was no longer observed after addition of β-glucuronidase to the reaction (Figure 2, lower panel). For a comparative activity analysis, the glucuronidation activities of both cell lines were initially examined using a variety of commonly-tested non-carcinogenic substrates, including 4-nitrophenol, 4-methylumbelliferone and 1-naphthol, with the relative UGT1A10 enzymatic activity normalized according to UGT1A10 cell line expression as determined by Western blot analysis against a known UGT1A1 standard (Figure 1C). For all substrates tested, the glucuronidation activity of homogenates from the polymorphic UGT1A10^{139Lys} cell line was significantly less ($p < 0.005$ in all cases) than that observed for the wild type UGT1A10^{139Glu} isoform (Table 2). Between 2.1- and 2.9-fold less activity was observed for UGT1A10^{139Lys} cell homogenates against all substrates tested. No glucuronidation activity was observed for untransfected HK293 cell homogenates for any substrate examined in this study (data not shown).

To assess whether these differences in activity were also manifested against

PAHs, kinetic analysis was performed against 1-OH-pyrene as well as four BaP metabolites, BPD, 3-OH-BaP, 7-OH-BaP, and 9-OH-BaP (Table 3). Differences in glucuronidation kinetics were observed between homogenates from the UGT1A10^{139Glu} and UGT1A10^{139Lys} cell lines against all substrates tested. A significantly (p<0.01) higher V_{max}/K_m was observed for homogenates from wild-type UGT1A10^{139Glu} cells against all four BaP metabolites tested. A similarly significant (p<0.05) increase in V_{max}/K_m was observed for homogenates from wild-type UGT1A10^{139Glu} cells against 1-OH-pyrene. Significantly lower apparent K_m values were observed for homogenates from wild-type UGT1A10^{139Glu} cells against 1-OH-pyrene ($p<0.05$) and 3-OH-BaP ($p<0.01$). Interestingly, the apparent K_m observed in the present study for homogenates from the wild-type UGT1A10^{139Glu} cell line against racemic BPD (46.8 μM) was almost 4-fold lower than that observed for wild-type UGT1A10-over-expressing baculosomes in previous studies (Fang et al, 2002).

Discussion

UGT1A10 is an extra-hepatic enzyme that is expressed in tissues of the digestive tract (Strassburg et al, 1999) and aerodigestive tract (Zheng et al, 2002). UGT1A10 has been implicated in the glucuronidation of several important carcinogens that likely play an important role in cancer initiation at these sites, including PAHs like BaP-7,8-dihydrodiol, precursor to the ultimate carcinogenic metabolite of BaP, BaP-7,8-dihydrodiol-9,10-epoxide (Fang et al, 2002), and 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhiP), the reactive intermediate of PhiP, a heterocyclic amine found in abundance in char-broiled meats (Malfatti and Felton, 2004). Previous reports identified two missense polymorphisms for UGT1A10, one at codon 139 that results in a non-conservative change in amino acids (Glu>Lys), and another at codon 244 that results in a conservative (Leu>Ile) amino acid change (Elahi et al, 2003). Both polymorphisms were somewhat prevalent (0.04-0.05) in African Amercians but much less prevaelent in other racial groups including Caucasians and Asians (<0.01 for both groups). In a small hospital-based case:control study of 230 African American subjects, only the codon 139 polymorphism was associated with orolaryngeal cancer risk, with subjects with the UGT1A10^{139Lys} variant exhibiting a significantly decreased risk for orolaryngeal cancer (Elahi et al, 2003).

The purpose of the present study was to further evaluate the role of UGT1A10 as a potentially important enzyme in the metabolism of PAHs and examine the effects of the UGT1A10 codon 139 polymorphism on UGT1A10 activity against the same PAHs *in vitro*. Results presented here clearly demonstrate that of all the UGTs examined,

UGT1A10 exhibits the highest glucuronidation activity against the various PAHs tested. These results are consistent with the high relative activity previously observed for UGT1A10 against both the (+) and (-) isomers of BPD (Fang et al, 2002). This suggests that UGT1A10 could be playing a major role in the detoxification of PAHs in important target sites including tissues within the digestive and aerodigestive tracts, where exposure to PAHs via char-broiled meats or tobacco smoke may be important in cancer induction.

Results from this study also demonstrated detectable levels of expression of UGT1A10 in lung tissue, indicating that UGT1A10 may also be playing a role in the detoxification of tobacco smoke PAHs within the lung. The fact that UGT1A10 was not observed to be expressed in lung tissue in previous studies is likely due to differences in assay sensitivity, since previous studies were performed using multiplex RT-PCR using β-actin as an internal control for amplification (Zheng et al, 2002). Although excellent for quality control, the use of multiple primers for the amplification of multiple genes in single PCR reactions can affect overall amplification sensitivity and can interfere with the amplification of low-copy number mRNAs (Markoulatos et al, 2002), a possibility for UGT1A10 in lung. Alternatively, it is possible that UGT1A10 may be localized to different regions within the lung, and that the lung specimens examined in the two studies were from different lung sites, or that UGT1A10 may have been differentially induced in the lungs from the different individuals from whom the specimens were obtained. Future studies will be necessary to evaluate these possibilities.

Results from the present study demonstrate that the UGT1A10^{139Lys} polymorphic variant exhibits significantly decreased glucuronidation activity against several PAHs

including metabolites of BaP as well as 1-OH-pyrene *in vitro*. The decreased activity observed for UGT1A10^{139Lys} cell homogenates against these PAHs was reflected by significantly decreased V_{max}/K_m and, in some cases, significantly higher apparent K_m, as compared to UGT1A10^{139Glu} cell homogenates. Similarly significant decreases in glucuronidation activity were observed for UGT1A10^{139Lys} cell homogenates against all non-carcinogenic aglycones examined in this study. The fact that there were differences in the apparent K_m for the wild-type UGT1A10 against BPD in the present study versus previous studies (Fang et al, 2002) is likely due to the fact that UGT1A10-over-expressing baculosomes were used in previous studies while a UGT1A10-over-expressing cell line was used in the present analysis. Recent studies have suggested that UGT1A10 activity is phosphorylation-dependent, potentially involving kinases that may not be present or as active in insect cells versus human cells (Basu et al, 2004).

Together, these results suggest that the non-conservative amino change of Glu to Lys at codon 139 renders UGT1A10 less active. Interestingly, this decrease in glucuronidation activity for the UGT1A10^{139Lys} variant is not consistent with the protective effect observed for this allele in orolaryngeal cancer case:control studies (Elahi et al, 2003). One possibility that could explain this is that the two variants may have different optimal assay conditions *in vitro*. Another possibility is that the previous case:control study was small (115 individually-matched African American case:control pairs) and that the association observed was an aberration. However, credence to the results of this study was provided by the fact that an association with orolaryngeal cancer risk was not observed with the more conservative codon 244 missense UGT1A10 polymorphism examined in the same population. The most likely possibility

that could explain the differences observed between these molecular epidemiologic studies and the functional studies outlined in this report is that the UGT1A10^{139Lys} allelic variant may be linked by haplotype to one or more other genetic variations within the UGT1A loci that not only compensates for the detrimental effect on UGT1A10 function by the presence of the codon 139 Lys residue but serves to provide an overall protective effect against orolaryngeal cancer risk. A haplotype analysis of potential variants within the UGT1A10 coding region was performed in previous studies, where the UGT1A10-specific exon 1 was screened for polymorphisms in a total of 102 subjects (53 Caucasians and 49 African Americans; Elahi et al, 2002). The allelic variant encoding the UGT1A10^{139Lys} isoform (termed UGT1A10*2) was not observed to be linked to any other UGT1A10 exon 1 polymorphisms in these studies. Alternatively, linkage could be with variants within the UGT1A10 promoter region, sequences that were not examined in these previous studies, or in linkage with other genetic variations within the UGT family 1A loci that somehow affect UGT1A10 expression. Another possibility is that, the UGT1A10 codon 139 polymorphism may be linked to functionally-relevant polymorphism(s) in another family 1A UGT that is important in orolaryngeal cancer risk. Previous studies have demonstrated that function-altering polymorphisms in the UGT1A7 gene are strongly associated with tobacco smoking-related orolaryngeal cancer risk in both Caucasians and African Americans (Zheng et al, 2001). Like UGT1A10, UGT1A7 was also shown to be the only other UGT to be well-expressed in all aerodigestive tract tissues examined (Zheng et al, 2002) and, as described in this and previous reports (Fang et al, 2002; Guillemette et al, 2000), UGT1A7 exhibits glucuronidating activity against various PAHs including BPD. It is not yet known

whether a genetic linkage exists between the UGT1A10 codon 139 polymorphism and polymorphisms in other regions within the UGT family 1A loci including UGT1A17. Studies attempting to identify UGT1A10 codon 139-inclusive haplotypes within the UGT family 1A locus by large-scale sequencing analysis of individuals with a UGT1A10*2 allele are currently in progress.

In summary, UGT1A10 is the most active UGT in the glucuronidation of various PAHs and is expressed in various target tissues where PAH exposure may be important in cancer induction, including tissues within the digestive and aerodigestive tracts and potentially within the lung. Further investigations will be required to fully elucidate the mechanism underlying the observed protective phenotype in orolaryngeal cancer risk associated with the functionally less-active UGT1A10^{139Lys} polymorphic variant.

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Footnotes

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Legends for Figures

Fig. 1. Analysis of UGT1A expression. (A) Western blot analysis of UGT1A protein levels in protein lysates from the individual UGT1A-over-expressing HK293 cell line homogenates or UGT1A-over-expressing baculosomes used in the glucuronidation activity analysis against select PAHs. To obtain a single blot with densitometric readings on the linear part of the curve for all family 1A UGTs, varying amounts of total protein were loaded as indicated. The relative densitometric values are also given for the representative blot shown setting the UGT1A1 standard to 1.0. *Fold-difference values are the average of three independent Western blot experiments. The relative expression of each UGT1A is shown with the level of UGT1A3 protein arbitrarily set to 1.0. (B) RT-PCR of total RNA from normal human lung tissue from a single subject. Lanes 1, 3 and 5, minus RNA negative control; lanes 2, 4 and 6, +lung RNA. Lanes 1 and 2, RT-PCR with β-actin primers; lanes 3 and 4, RT-PCR with UGT1A10 exon 1 primers; lanes 5 and 6, multiplex RT-PCR with both β-actin and UGT1A10 exon 1 primers. (C) Western blot analysis of UGT1A10 protein from UGT1A10^{139Glu}- and UGT1A10^{139Lys}-over-expressing cell lines. Equal amounts (40 μg) of protein from each of the indicated cell protein lysates were loaded to each lane. β-Actin was used as an internal control for protein loading for each lane. The relative ratios of UGT1A10:β-actin levels are shown under the corresponding lanes for each of the UGT1A10-over-expressing cell lines, with the UGT1A10:β-actin ratio for the UGT1A10^{139Glu}-over-expressing cell protein lysate designated as 1.0 as reference. One hundred ng of UGT1A10 protein standard (from

Gentest) was loaded as a gel-loading reference.

Fig. 2. HPLC analysis of 4-nitrophenol glucuronide formation using UGT1A10-over-expressing cell line homogenates. Top panel, UGT1A10^{139Glu}- over-expressing cell homogenate; middle panel, UGT1A10^{139Lys}-over-expressing cell homogenate; bottom panel, UGT1A10^{139Glu}-over-expressing cell homogenate with treatment with β-glucuronidase.

Table 1

Kinetic analysis of individual human UGT enzymes against various PAHs[a]

UGT	3-OH-BaP		7-OH-BaP		9-OH-BaP		1-OH-Pyrene	
	K_m[c] (μM)	V_{max}/K_m[d] (μl·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m[d] (μl·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m[d] (μl·min^{-1}·mg^{-1})	K_m (μM)	V_{max}/K_m[d] (μl·min^{-1}·mg^{-1})
1A1	55.6 ± 33.7	8.2 ± 7.1	281 ± 9	3.3 ± 0.2	209 ± 5	2.7 ± 0.3	96.3 ± 23.3	1.3 ± 0.1
1A3	348 ± 64	17.5 ± 3.5	50.1 ± 7.9	76.5 ± 11.6	208 ± 13	57.7 ± 4.0	1526 ± 939	0.35 ± 0.05
1A4	ND		ND		ND		ND	
1A6	ND		ND		ND		113 ± 21.3	537 ± 18.1
1A7	115 ± 23	29.2 ± 5.1	129 ± 6	35.9 ± 1.5	30.1 ± 2.1	3.2 ± 0.3	34.2 ± 1.7	21.4 ± 4.1
1A8	234 ± 98	375 ± 125.3	217 ± 71	25.7 ± 11.7	615 ± 106	29.6 ± 2.2	854 ± 117	159 ± 6.8
1A9	63.5 ± 7.0	335 ± 20.9	62.1 ± 14.1	201 ± 4.6	125 ± 65	176 ± 11.9	29.9 ± 2.8	27.9 ± 1.6
1A10	9.7 ± 1.2	2557 ± 124.3	9.8 ± 0.5	2633 ± 81	38.2 ± 6.2	1817 ± 58.6	11.3 ± 3.3	7965 ± 2100
2B4	ND		ND		ND		ND	
2B7[b]	583 ± 25	NP	190 ± 90	NP	63.3 ± 17.1	NP	2869 ± 3.3	NP
2B15[b]	497 ± 4.3	NP	542 ± 55	NP	91.8 ± 25.5	NP	169 ± 12.4	NP
2B17[b]	295 ± 88	NP	1588 ± 314	NP	481 ± 51	NP	87.0 ± 19.5	NP

[a] All reactions were performed using cell homogenate or baculosomal preparations for individually over-expressed UGT enzymes, and incubations were for 1 h or [b] 30 min.

[c] Kinetic data are reported as mean ± standard deviation for three independent experiments. Km, apparent Km. ND, not detected.

[d] V_{max} values are adjusted per mg of the corresponding UGT1A protein as determined by Western blot analysis. NP, not performed.

Table 2

Glucuronidation of non-carcinogen substrates by UGT1A10^{139Glu}- and UGT1A10^{139Lys}-over-expressing cell homogenates

	enzymatic activity (nmol·min^{-1}·mg^{-1} UGT1A10 protein)[a]	
substrate	**UGT1A10^{139Glu}**	**UGT1A10^{139Lys}**
4-nitrophenol	348 ± 39	122 ± 8[b]
4-methylumbelliferone	669 ± 24	290 ± 59[b]
1-naphthol	365 ± 19	172 ± 11[b]

[a] Data reported as the mean ± standard deviation of three experiments. Shown are glucuronidation rates normalized by the relative levels of UGT expression in the individual UGT1A10-over-expressing cell homogenates as determined by Western blot analysis. Glucuronidation assays were performed using 0.5 mg UGT1A10-over-expressing cell homogetate for a 1 h incubation at 37°C. Aglycone concentrations were 1mM.

[b] Significant ($p < 0.005$) differences in glucuronidation activity were observed between UGT1A10^{139Glu}- versus UGT1A10^{139Lys}-over-expressing cell homogenates.

Table 3

Kinetic analysis of glucuronide formation for BaP metabolites for UGT1A10^{139Glu}- and UGT1A10^{139Lys}-over-expressing cell homogenates[a]

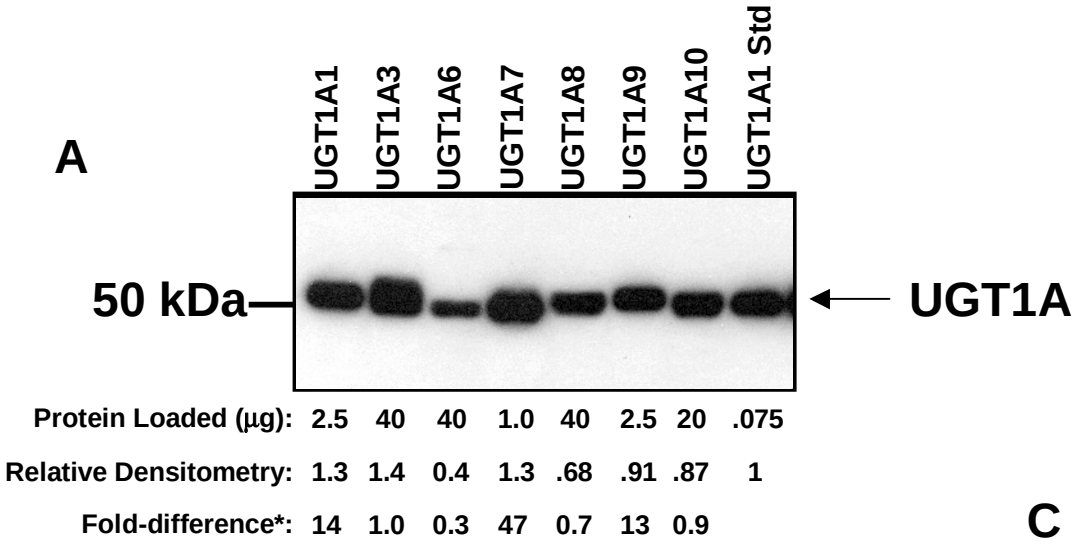
	UGT1A10^{139Glu}			UGT1A10^{139Lys}		
substrate	Km (μM)	Vmax (nmol min^{-1} mg^{-1})[b]	Vmax/Km (μl min^{-1}mg^{-1})[b]	Km (μM)	Vmax (nmol min^{-1} mg^{-1})[b]	Vmax/Km (μl min^{-1} mg^{-1})[b]
BPD	46.8 ± 4.2	7.7 ± 0.4	165 ± 6.5	53.2 ± 10.9	3.9 ± 0.2[c]	73.3 ± 12.7[c]
3-OH-BaP	9.7 ± 1.2	24.8 ± 1.9	2557 ± 124.3	15.4 ± 1.6[c]	17.7 ± 1.2[c]	1149 ± 41.1[c]
7-OH-BaP	9.8 ± 0.5	25.8 ± 0.7	2633 ± 81	9.6 ± 0.5	13.2 ± 0.8[c]	1375 ± 16.6[c]
9-OH-BaP	38.2 ± 6.2	69.4 ± 9.2	1817 ± 58.6	48.2 ± 3.6	44.8 ± 2.4	929 ± 20.8[c]
1-OH-pyrene	11.3 ± 3.3	90.0 ± 2.4	7965 ± 2100	14.7 ± 3.9[d]	50.4 ± 3.1[c]	3429 ± 770[d]

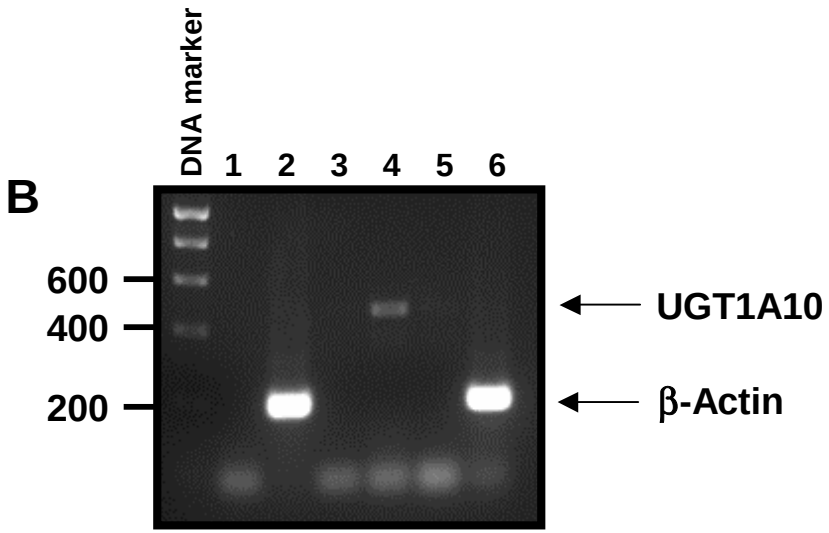
[a] All reactions were performed using 0.5 mg UGT1A10-over-expressing cell homogenate, with incubations performed for 1 h. Kinetic data are reported as mean ± standard deviation for three independent experiments. Km, apparent Km. ND, not detected.

[b] V_{max} values are adjusted per mg of the corresponding UGT1A protein as determined by Western blot analysis.

[c] Significant ($p < 0.01$, or [d] $p < 0.05$) increase in kinetic parameter observed for homogenates from UGT1A10^{139Glu}- versus UGT1A10^{139Lys}-over-expressing cells.

Figure 1





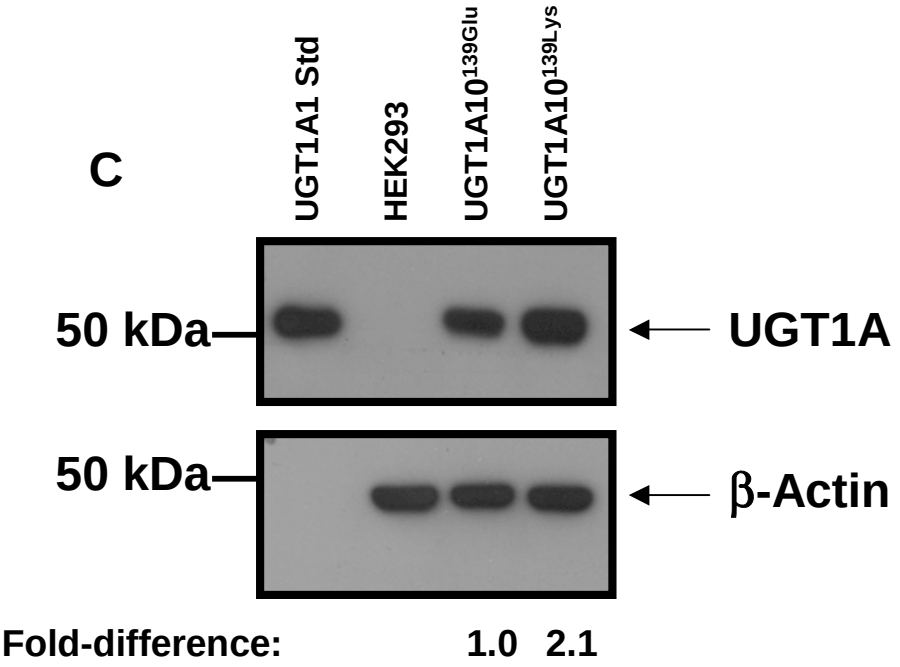


Figure 2