Regulation and function of family 1 and family 2 UDP-glucuronosyltransferase genes (*UGT1A*, *UGT2B*) in human oesophagus

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Human UDP-glucuronosyltransferases (UGTs) are expressed in a tissue-specific fashion in hepatic and extrahepatic tissues [Strassburg, Manns and Tukey (1998) J. Biol. Chem. **273**, 8719–8726]. Previous work suggests that these enzymes play a protective role in chemical carcinogenesis [Strassburg, Manns and Tukey (1997) Cancer Res. **57**, 2979–2985]. In this study, *UGT1* and *UGT2* gene expression was investigated in human oesophageal epithelium and squamous-cell carcinoma in addition to the characterization of individual UGT isoforms using recombinant protein. UGT mRNA expression was characterized by duplex reverse transcriptase-PCR analysis and revealed the expression of UGT1A7, UGT1A8, UGT1A9 and UGT1A10 mRNAs. UGT1A1, UGT1A3, UGT1A4, UGT1A5 and UGT1A6 transcripts were not detected. UGT2 expression included UGT2B7, UGT2B10 and UGT2B15, but UGT2B4 mRNA was absent. UGT2 mRNA was present at significantly lower levels than UGT1 transcripts. This observation was in agreement with the analysis of catalytic activities in oesophageal microsomal protein, which was characterized by high glucuronidation rates for phenolic xenobiotics, all of which are classical UGT1 substrates. Whereas UGT1A9 was not regulated, differential regulation of UGT1A7 and UGT1A10 mRNA was observed between normal oesophageal epithelium and squamous-cell carcinoma. Expression and analysis *in vitro* of recombinant UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15 demonstrated that UGT1A7, UGT1A9 and UGT1A10 catalysed the glucuronidation of 7-hydroxybenzo(α)pyrene, as well as other environmental carcinogens, such as 2-hydroxyamino-1-methyl-6-phenylimidazo-(4,5-β)-pyridine. Although UGT1A9 was not regulated in the carcinoma tissue, the five-fold reduction in 7-hydroxybenzo(α)pyrene glucuronidation could be attributed to regulation of UGT1A7 and UGT1A10. These data elucidate an individual regulation of human *UGT1A* and *UGT2B* genes in human oesophagus and provide evidence for specific catalytic activities of individual human UGT isoforms towards environmental carcinogens that have been implicated in cellular carcinogenesis.

Key words: carcinogenesis, diet, heterocyclic amine, metabolism, polycyclic aromatic hydrocarbon.

INTRODUCTION

UDP-glucuronosyltransferases (UGTs) are a superfamily of proteins located in the endoplasmic reticulum, which perform a key process in endobiotic and xenobiotic metabolism [1,2]. UGTs catalyse the conversion of lipophilic molecules into more polar, and thus hydrophilic, glucuronides, thereby facilitating the subsequent elimination via bile, faeces and urine. A diverse array of substrates are targeted for glucuronidation, including phenolics, steroid hormones and carcinogens such as primary amines and benzo(α)pyrene metabolites, as well as many therapeutically used tertiary amines, and non-steroidal anti-inflammatory and coumarin drugs. Clearly, UGTs play an important role in the detoxification of endogenous, dietary and clinically administered agents as well as environmentally present chemicals that have been shown to lead to a toxic or carcinogenic episode [3].

Based on primary amino acid sequences, the UGTs have been divided into two families of proteins termed UGT1 and UGT2 [4]. The *UGT2* genes are encoded as individual structural genes on chromosome 4 [5]. From human liver, four *UGT2* cDNAs have been cloned and characterized: UGT2B4 [6], UGT2B7 [7], UGT2B10 [8] and UGT2B15 [9,10]. In contrast, the human *UGT1* genes are encoded by a single locus on chromosome 2. The *UGT1A* locus encodes nine functional exon-1 cassettes (*UGT1A1*, *UGT1A3–10*) and three non-functional exon-1 sequences (*UGT1A2*, *UGT1A11* and *UGT1A12*). The linear array of 12 exon-1 sequences is followed by common exons 2–5 [2]. *UGT1A* gene products are generated by a strategy of exon sharing, which combines the individual first exons and common exons 2–5 to form unique UGT transcripts [11]. The analysis of *UGT1A* gene products has led to the cloning of cDNAs that encode UGT1A1 [12], UGT1A3 [13], UGT1A4 [12], UGT1A6 [14] and UGT1A9 [15] from human liver. A tissue-specific expression of strictly extrahepatic UGT1A transcripts has been identified for UGT1A7 in gastric tissue [16,17], UGT1A8 in colon tissue [18,19] and UGT1A10 in gastric, biliary and colon tissue [16–19].

Regulation of the *UGT1A* locus therefore appears to be designed to reflect specific metabolic requirements defined by anatomical location. The analysis of recombinant UGT cDNAs has shown that UGT1A proteins favour xenobiotic substrates, including a wide range of phenolic compounds, flavones and amines [12,13,18,20,21]. Some of these UGTs have unique catalytic activities catalysed by the bilirubin UGT1A1 [22], tertiary amine UGT1A4 [23] and oestrone UGT1A3 and UGT1A10 [13,18]. Conversely, recombinant UGT2B proteins exhibit a preference for endobiotic substrates, including hydroxylated steroids and bile acids [4,6–9]. Individual isoforms such as UGT2B7 and UGT2B15 also demonstrate catalytic activity towards some xenobiotic substrates [10,24].

The number of UGT proteins and the range of target substrates indicate the significance of glucuronidation in human metabolism. UGT1A proteins have been linked to fatal genetic abnormalities such as Crigler–Najjar type-I disease [2]. They

Abbreviations used: UGT, UDP-glucuronosyltransferase; RT, reverse transcriptase; N-hydroxy-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo-(4,5-β)-pyridine; DRT-PCR, duplex RT-PCR; PhIP, 2-amino-1-methyl-6-phenylimidazo-(4,5-β)-pyridine.

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have also been identified as hepatocellular autoantigens in autoimmune hepatitis [25] and viral hepatitis D [25,26], and implicated in human hepatocarcinogenesis [27] and gastric carcinogenesis [16]. Their ability to glucuronidate carcinogenic compounds, such as primary amines [23] and benzo(α)pyrene metabolites [13,18,19], suggests that UGT may play an important role in cellular protection against oxidative or chemical genotoxicity [16,27–30]. Dietarily borne human carcinogens enter the body via the gastrointestinal tract and come into contact with mucosal tissue of the oesophagus prior to downstream resorption and subsequent metabolism. Epidemiological evidence suggests a link between mutagen exposure and different gastrointestinal cancers in humans [31–33]. This association is well documented in squamous oesophageal carcinoma, which has a significantly increased occurrence in individuals who consume tobacco products [33]. To elucidate potential biological mechanisms capable of influencing this disease, data on the regulation and function of classical detoxifying enzymes such as the UGTs may be helpful. However, regulation of *UGT1A* and *UGT2B* gene products in human oesophagus has not been studied in detail.

An analysis of *UGT1A* and *UGT2B* gene expression in human oesophageal mucosa and corresponding squamous-cell carcinoma was evaluated. In the present study we examined *UGT1A* and *UGT2B* gene expression in combination with catalytic activity determinations using oesophageal microsomal protein and recombinant UGTs.

MATERIALS AND METHODS

Tissue samples

Oesophageal tissue was obtained from two male German patients with squamous-cell carcinoma of the middle third of the oesophagus (sample 1 age, 57; sample 2 age, 68) undergoing oesophagectomy at the University of Hannover School of Medicine Medical Centre, Hannover, Germany. Corresponding normal oesophageal mucosa was sampled near the distal resection margin. Squamous-cell carcinoma and normal squamous-cell epithelium were confirmed by microscopy. The samples were free of other pathological findings such as inflammation, ulcer or necrosis. Both patients had a history of cigarette smoking but did not receive radiation or chemotherapy prior to surgery. Samples were frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

RNA isolation

Approximately 75 mg of frozen oesophageal tissue was pulverized in a mortar filled with liquid nitrogen. Tissue powder was immediately lysed in acidic phenol/guanidinium isothiocyanate solution (TriPure; Boehringer Mannheim, Indianapolis, IN, U.S.A.) and RNA extracted as outlined previously [17]. Concentrations were measured by absorbance at 260 and 280 nm and RNA samples were stored in water at −80 °C until further use. RNA was visualized by agarose-gel electrophoresis containing formaldehyde with subsequent acridine orange staining. $A_{260/280}$ measurement ratios were above 1.9.

Complementary DNA synthesis and documentation of DNA-free RNA

Total RNA (3 mg) was denatured for 10 min at 70 °C in the presence of 0.5 mg of oligo(dT) primer, and placed on ice. The reaction conditions were adjusted to 20 mM Tris/HCl (pH 8.4)/50 mM KCl/2.5 mM $MgCl_2$/10 mM dithiothreitol/0.5 mM of each dNTP in a total volume of 19 μl, which was incubated at 42 °C for 5 min. Synthesis was started by adding 200 units of reverse transcriptase (RT, Superscript II; Life Sciences, Gaithersburg, MD, U.S.A.). The final volume of 20 μl was incubated at 42 °C for 50 min and at 70 °C for 15 min, and placed on ice.

DNA contamination of RNA was excluded by RT-PCR. Amplification of human β-actin with the primers 5′-GGCGG-CACCACCATGTACCCT-3′ and 5′-AGGGGCCGGACTCG-TCATACT-3′ [27] spanning the exon 4–intron 5–exon 5 junction of human β-actin leads to a 202-bp product when a cDNA template is present, but results in a 312-bp product when a genomic DNA template is present. Both products can be clearly distinguished by 2% agarose-gel electrophoresis. All cDNAs in this study displayed a 202-bp product and were thus free of contaminating genomic DNA.

Isolation of microsomal protein from oesophageal tissue

Microsomal protein was isolated following standard published protocols [16]. Briefly, approximately 100 mg of oesophageal tumour and normal mucosa specimens were pulverized under liquid nitrogen, resuspended in 0.5 ml of 50 mM Tris/HCl, pH 7.4, containing 10 mM $MgCl_2$, and homogenized with a Potter-Elvehjem tissue grinder with 10 strokes at 3000 revs./min on ice. The tissue homogenate was centrifuged with 10000 ***g*** for 5 min at 4 °C and the supernatant collected. The pellet was suspended in 0.5 ml of buffer, centrifuged at 10000 ***g*** for 5 min and the supernatant collected. The combined supernatants were centrifuged at 150000 ***g*** for 60 min at 4 °C in a Beckman TL-100 ultracentrifuge and the pellet was suspended in 0.2 ml of 50 mM Tris/HCl, pH 7.4, containing 10 mM $MgCl_2$. After this procedure, the 150000-***g*** supernatant was free of UGT1A protein when examined by Western-blot analysis or for catalytic activity. Protein concentrations were determined by the method of Bradford [34]. Microsomal protein was stored at −80 °C.

Catalytic activity assay of human oesophageal microsomes and recombinant UGT proteins

The substrates 4-nitrophenol, 4-methylumbelliferone, vanillin, 2-hydroxybiphenyl, 4-*tert*-butylphenol, oestrone, 7-hydroxyflavone, hydrodeoxycholic acid, β-oestradiol, naringenin and 8-hydroxybenzo(α)pyrene were solubilized in methanol, and 7-hydroxybenzo(α)pyrene was resuspended in acetone. 7-Hydroxybenzo(α)pyrene and 8-hydroxybenzo(α)pyrene were obtained from the National Cancer Institute Chemical Carcinogen repository, Midwest Research Institute, Kansas City, MO, U.S.A.; all other substrates were from Sigma–Aldrich, St. Louis, MO, U.S.A. 2-Hydroxyamino-1-methyl-6-phenylimidazo-(4,5-β)-pyridine (N-hydroxy-PhIP) was purchased from Toronto Research Chemicals, Inc., Ontario, Canada. Catalytic activities of 25 μg of microsomal protein from human tissues were assayed in duplicate in the presence of 0.1 mM UDP-glucuronic acid/0.08 mg/ml phosphatidylcholine/0.04 μCi ^{14}C-labelled glucuronic acid/100 mM of test substrate in 50 mM Tris/HCl (pH 7.6)/10 mM $MgCl_2$ for 60 min at 37 °C. These conditions were optimized to provide saturating conditions in order to achieve V_{max} in every reaction. For analysis of glucuronidating activities of whole-cell extracts made from baculovirus-transfected Sf9 cells, 100 μg of total cell extract was employed. In addition, 0.2 μCi ^{14}C-labelled UDP-glucuronic acid was used along with 0.1 mM UDP-glucuronic acid, and the incubations carried out for 2 h. Following the incubations, protein was subsequently precipitated by the addition of ethanol followed by centrifugation. Lyophilized supernatants were resuspended in 50 μl of methanol and separated by TLC in n-butanol/acetone/acetic acid/water (3.5:3.5:1:2, by vol.).

^{14}C- Labelled glucuronides were detected by autoradiography. Glucuronide bands were then removed from the TLC plates. In every lane an identical length of TLC material was scraped to ensure a precise quantification, even in cases where the glucuronides produced smeared bands (compare Figure 3, below). Counts were then determined by liquid scintillation counting and specific catalytic activities calculated in terms of pmol of glucuronide formed/mg of protein per min, as described previously [35]. To determine whether microsomal preparations and recombinant proteins were adequately activated, treatment with detergents, including Triton X-100, Tween 20 and Nonidet P50, was performed at varying concentrations, which failed to enhance UGT catalytic activity.

Expression of UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15 in baculovirus

The cloning and expression of catalytically active *UGT1A7* and *UGT1A10* have recently been reported [18]. The full-length cDNA encoding *UGT2B15* [9] was cloned by screening a λ-Zap cDNA library (Stratagene, La Jolla, CA, U.S.A.) with rabbit *UGT2B13* cDNA [36] as a probe. The cloning of *UGT1A9* [15] was performed using specific oligonucleotide primers for RT-PCR amplification. A 5′ sense primer (5′-C GATCTAGAC TGC AGT TCT CTG ATG GCT T-3′) incorporated an *Xba*I restriction site (underlined) upstream of the cDNA's (GenBank accession no. S55985) ATG initiation codon, and a 3′ antisense primer (5′-TTT GGGATCCAC TTC TCA ATG GGT CTT GG-3′) with a *Bam*HI restriction site (underlined) downstream from the cDNA's TGA termination codon. The cloning of *UGT2B7* [7] was conducted using specific oligonucleotide primers and RT-PCR amplification. The UGT2B7 sense primer was 5′-AT TGCTCGAGG ATG GTC TGT GAA ATG G-3′ and contained an *Xho*I restriction site (underlined), whereas the UGT2B7 antisense primer was 5′-CTC AGGTACCAC TAA TCA TTT TTT CCC-3′ and contained a *Kpn*I restriction site (underlined) to allow for the directional cloning of the entire UGT2B7 open reading frame. Full-length cDNA and PCR clones were characterized by dideoxy sequencing. The sequences were in agreement with the published *UGT1A9* (Genbank accession no. S55985), *UGT2B15* (GenBank U98854) and *UGT2B7* (GenBank J05428) cDNAs. *UGT1A9*, *UGT2B7* and *UGT2B15* were then subcloned into the baculovirus transfer vector pBluebac4, and transfected into exponential-phase *Spodoptera Frugiperda* insect-cell (Sf9) monolayers as described previously [35]. *UGT1A9*-, *UGT2B7*- and *UGT2B15*-recombinant virus supernatant with a titre of $1–2 \times 10^8$ p.f.u./ml was generated and subsequently used to infect exponential-phase Sf9 cell monolayers at a multiplicity of infection of 10 together with previously reported *UGT1A7*- and *UGT1A10*-recombinant virus [18]. Cells were collected at 60 h post-infection and lysed with a Potter-Elvejhem tissue grinder in buffer containing 50 mM Tris/HCl (pH 7.4)/10 mM $MgCl_2$. Recombinant UGT cell lysates were stored at -70 °C until analysis, and used for UGT analysis as outlined above.

Duplex RT-PCR (DRT-PCR)

All UGT1A transcripts

The presence of UGT1A transcripts in total tissue RNA was detected by the amplification of a 487-bp fragment from the conserved *UGT1A* exons 2–5, using DRT-PCR as reported previously in detail [17]. In brief, amplification with UGT1A primers at concentrations of 2 μM each was first carried out for six cycles using a Perkin-Elmer 480 Thermocycler. After the addition of β-actin primers to a concentration of 0.4 μM, cycling was continued for a total of 32 cycles at 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min. PCR was preceded by a hot start of 3 min at 94 °C and followed by a 7-min elongation at 72 °C. DRT-PCR products were separated in a 2 % agarose gel stained with ethidium bromide. Polaroid (Cambridge, MA, U.S.A.) type-665 positive/negative film was used to quantify bands by laser densitometry on an LKB 2222-020 UltroScan XL densitometer (LKB, Bromma, Sweden). Arbitrary units were calculated relative to β-actin products according to the following formula: (mean peak area for UGT/mean peak area for β-actin) $\times$ 100 = relative arbitrary units. Independent and combined linear kinetics for both products during the amplification process were established as described previously in detail [17].

Exon-1-specific DRT-PCR

The *UGT1A* locus predicts the existence of nine proteins, termed UGT1A1 and UGT1A3–10. UGT1A2, UGT1A11 and UGT1A12 each lack an uninterrupted open reading frame and have therefore been identified as pseudogenes [2]. DRT-PCR detection of all nine UGT1A transcripts predicted by the human *UGT1A* locus was performed using nine exon-1-specific sense primers and two antisense primers located within exons 2–5 or within a common portion of the 3′ end of the first exons [17]. As already reported elsewhere [17], exon-specific primers were generated that lead to RT-PCR products of distinct molecular sizes: UGT1A1, 644 bp; UGT1A3, 483 bp; UGT1A4, 572 bp; UGT1A5, 659 bp; UGT1A6, 562 bp; UGT1A7, 754 bp; UGT1A8, 514 bp; UGT1A9, 392 bp; and UGT1A10, 478 bp. Co-amplification of UGT1A first exon and β-actin sequences was performed using three cycling protocols: UGT1A1 and UGT1A6, 94 °C (1 min), 59 °C (1 min) and 72 °C (1 min); UGT1A3–5, 94 °C (1 min), 56 °C (1 min) and 72 °C (1 min); and UGT1A7–10, 94 °C (1 min), 64 °C (1 min) and 72 °C (1 min). Each protocol was preceded by a 3-min incubation of the reaction mixture at 94 °C and followed by a 7-min elongation at 72 °C. The specificity and kinetics of this assay have previously been documented in detail [17,18,27]. Experiments were performed in duplicate and controls without cDNA, primers or thermophilic polymerase included. Quantification of products by laser densitometry was performed as described above.

UGT2B-specific DRT-PCR

Primer pairs were generated for UGT2B4, UGT2B7, UGT2B10 and UGT2B15. Cross reactivity was excluded using sequence alignments and PCGene software (Oxford Molecular, Campbell, CA, U.S.A.), as well as a computerized databank search using the BLASTN software (GenBank). Base-pair numbers and sequence data refer to the following accession numbers (acn.) deposited in GenBank: UGT2B4 sense primer (acn. Y00317, bp 290–314), 5′-GAGTTTGAGGATATTATCAAGCAGC-3′, UGT2B4 antisense primer (acn. Y00317, bp 570–550), 5′-TAGCCAGGAGAGAAGCGAGGC-3′; UGT2B7 sense primer (acn. J05428, bp 1424–1445), 5′-CAAAGGAHCTAAACAC-CTTCGG-3′, UGT2B7 antisense primer (acn. J05428, bp 1830–1809), 5′-CCGTAGTGTTTTCTTCATTGCC-3′; UGT2B10 sense primer (acn. X63359, bp 1679–1702), 5′-GCTCACTT-ATCCTATCTCCTTGGC-3′, UGT2B10 antisense primer (acn. X63359, bp 2066–2047), 5′-GGGTAGAAGGATTGGATGCC-3′; UGT2B15 sense primer (acn. U08854, bp 1612–1636), 5′-AGTTATATCAAAGCCTGAAGTGG-3′, UGT2B15 antisense primer (acn. U08854, bp 1941–1921), 5′-TGTGCACAACG-AAGGGTTAGC-3′. *UGT2B* cDNA was co-amplified with β-

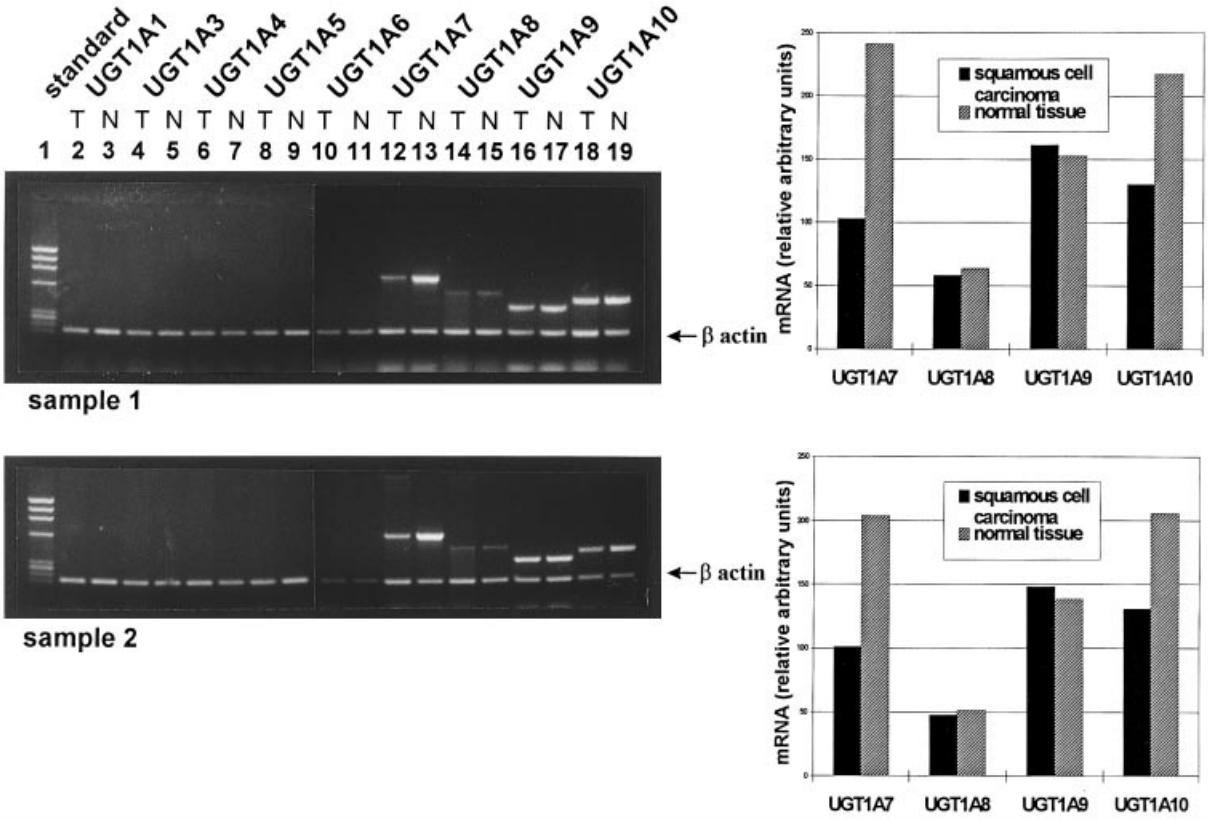

Figure 1 Expression of the *UGT1A* gene locus in human oesophagus and squamous-cell carcinoma of the oesophagus detected by DRT-PCR

In both samples expression of *UGT1A7*, *UGT1A8*, *UGT1A9* and *UGT1A10* was detected, all other isoforms were absent. Comparisons of carcinoma (T) and normal (N) tissue from the same patient exhibited differential downregulation of UGT1A7 mRNA and, to a lesser degree, of UGT1A10 mRNA. Quantification (right panels, histograms) was performed by laser densitometry as outlined in the Materials and methods section. Standard, *Hae*III digest of ΦX174 DNA.

actin cDNA in a starting volume of 92 μl containing 10 mM KCl, 20 mM Tris/HCl (pH 8.8), 10 mM ammonium sulphate, 2 mM magnesium sulphate, 1% Triton X-100, 0.2 mM each dNTP and 2 μM of UGT2B primers. After a hot start at 94 °C for 3 min, six cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s were run on a Perkin-Elmer GeneAmp PCR 2400 system. The same β-actin primers used for UGT1A DRT-PCR were added to 0.4 μM each and cycling was resumed for a total of 32 cycles. Specificity of this assay was determined by PCR using all four primer pairs on each cloned *UGT2B4*, *UGT2B7*, *UGT2B10* and *UGT2B15* template cDNA to exclude cross reactivities. In addition, UGT2B DRT-PCR was performed using hepatic RNA known to express these gene products. PCR products of the expected sizes were generated: UGT2B4, 281 bp; UGT2B7, 407 bp; UGT2B10, 388 bp; and UGT2B15, 330bp. DRT-PCR products were additionally analysed by Southern blot and direct dideoxy sequencing. Linear amplification kinetics were established for the dual products by termination of PCR reactions after 28, 30, 32, 34 and 36 cycles, as previously reported [17]. Products were quantified by laser densitometry and arbitrary units calculated as described for UGT1A DRT-PCR.

Western-blot analysis of microsomal protein and recombinant UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15

Microsomal protein (25 μg) or 20 μg of total Sf9 cell lysate expressing UGT1A1 [25], UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15 was boiled for 90 s in loading buffer [2% SDS/62.5 mmol/l Tris/HCl (pH 6.8)/10% glycerol/0.001% Bromophenol Blue) with β-mercaptoethanol and resolved by 10% SDS/PAGE prior to electrotransfer on to nitrocellulose membrane. Immunodetection was performed following published protocols [25]. UGT1A protein was detected using a rabbit anti-UGT1A anti-C-terminal-peptide antiserum diluted 1:3000 [18]. Detection of both UGT1A and UGT2B recombinant proteins was achieved with a sheep anti-rabbit UGT antibody with specificity for both UGT1A and UGT2B proteins [35]. Visualization was carried out with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:4500.

RESULTS

Tissue-specific expression of the *UGT1A* locus in human oesophagus and differential regulation in squamous-cell carcinoma

Expression of all *UGT1A* gene products predicted by the human *UGT1A* locus was analysed by DRT-PCR in two oesophageal normal/carcinoma tissue sample pairs (Figure 1). Both samples were characterized by the exclusive expression of UGT1A7, UGT1A8, UGT1A9, and UGT1A10, whereas UGT1A1, UGT1A3, UGT1A4, UGT1A5 and UGT1A6 mRNAs were not detected. This finding established the unique expression of the UGT1A7–10 cluster of extrahepatic *UGT1A* gene products in human oesophagus. Comparison between healthy oesophageal

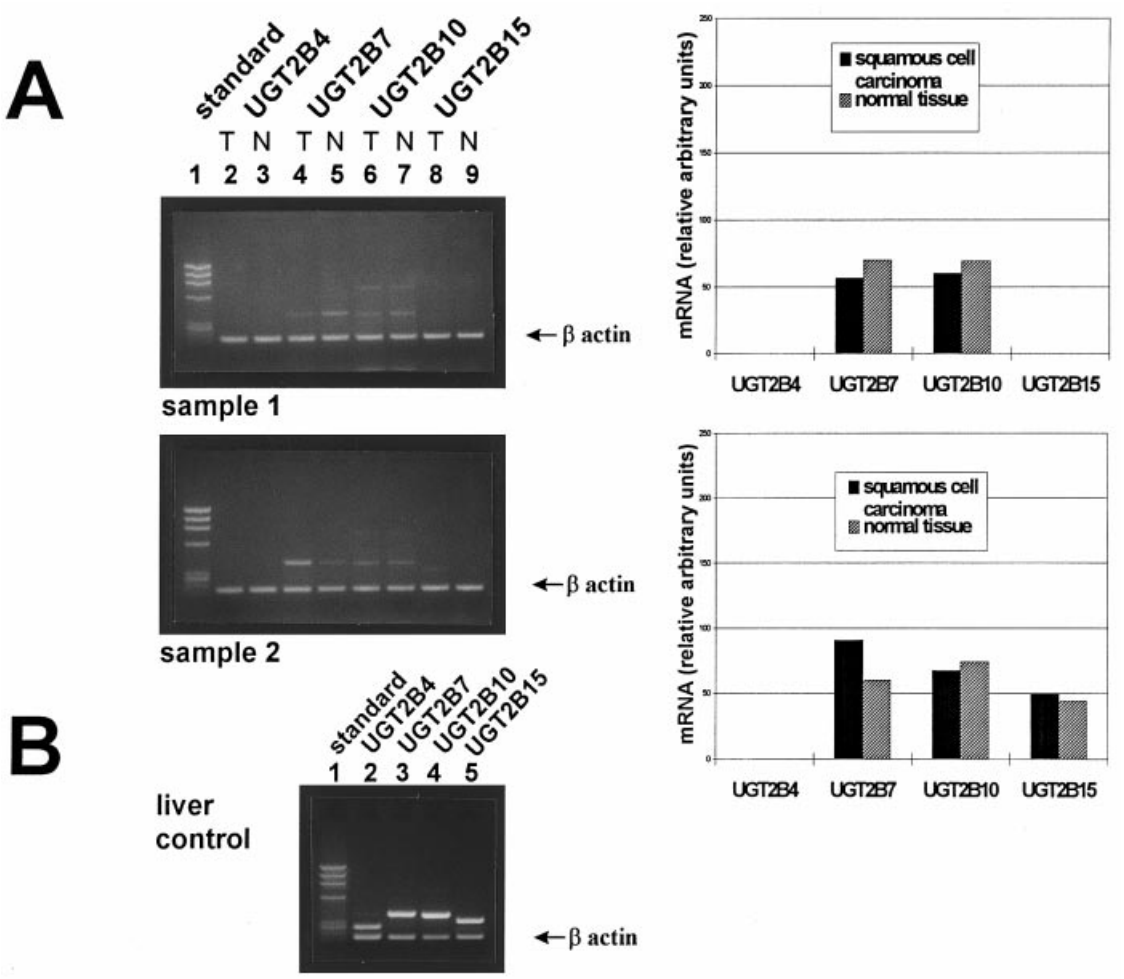

Figure 2 Expression of *UGT2B* genes in human oesophagus and squamous-cell carcinoma of the oesophagus detected by DRT-PCR

(**A**) UGT2B7 and UGT2B10 were expressed in both samples, UGT2B15 was detected at low levels in only sample 2. No UGT2B4 mRNA was detectable in either sample. Overall UGT2B mRNA levels were lower than UGT1A mRNA (see Figure 1), and differential UGT2B regulation between carcinoma (T) and normal (N) tissue was not observed. (**B**) UGT2B4, UGT2B7, UGT2B10 and UGT2B15 mRNAs were each detected at higher levels with the same assay using hepatic-tissue RNA. Standard, *Hae*III digest of ΦX174 DNA.

mucosa and squamous-cell carcinoma revealed substantial differential regulation for the UGT1A7 and UGT1A10 transcripts. The relative expression levels of UGT1A8 and UGT1A9 between different tissues were similar, with UGT1A9 being much more abundant. This finding was consistent between the two paired tissue samples. Whereas it has been very difficult to obtain additional samples of oesophageal tumour for more complete statistical analysis (the present samples were collected over a 12-month period), the data indicate tissue-specific regulation of the human *UGT1A* locus in oesophagus, as well as differential regulation of UGT1A7 and UGT1A10 expression in tumour tissue.

Tissue-specific expression of UGT2B4, UGT2B7, UGT2B10 and UGT2B15 in human oesophagus and squamous-cell carcinoma

UGT2B expression was also established by isoform specific DRT-PCR (Figure 2). In both oesophageal tissue samples, UGT2B7 and UGT2B10 were detected in normal and cancer tissue. UGT2B4 was absent from both samples, whereas UGT2B15 was only found in sample 2. Quantification of UGT2B transcripts demonstrated no regulation of individual UGT2B transcripts between normal and carcinoma tissue. The overall expression of UGT2B mRNA was lower than that of UGT1A mRNA in the same samples. A control experiment demonstrated the detection of high levels of UGT2B4, UGT2B7, UGT2B10 and UGT2B15 mRNAs in liver tissue by DRT-PCR (Figure 2B).

Differential UGT activity in oesophageal mucosa and squamous-cell carcinoma

Using normal oesophageal mucosa microsomes (sample 2) specific UGT activities were determined for a panel of 12 substrates, including phenolic, flavone, steroid and hydroxylated benzo(α)pyrene compounds (Figure 3 and Table 1). The highest activities were found for phenolic substrates such as 4-methylumbelliferone, 2-hydroxybiphenyl, 4-*tert*-butylphenol and vanillin, as well as for 7-hydroxybenzo(α)pyrene. These xenobiotic compounds are all classical substrates for UGT1A proteins. Interestingly, the specific activity for hydrodeoxycholic acid was the lowest activity determined. Hydrodeoxycholic acid conjugation was found to be 53-fold lower than 4-methylumbelliferone

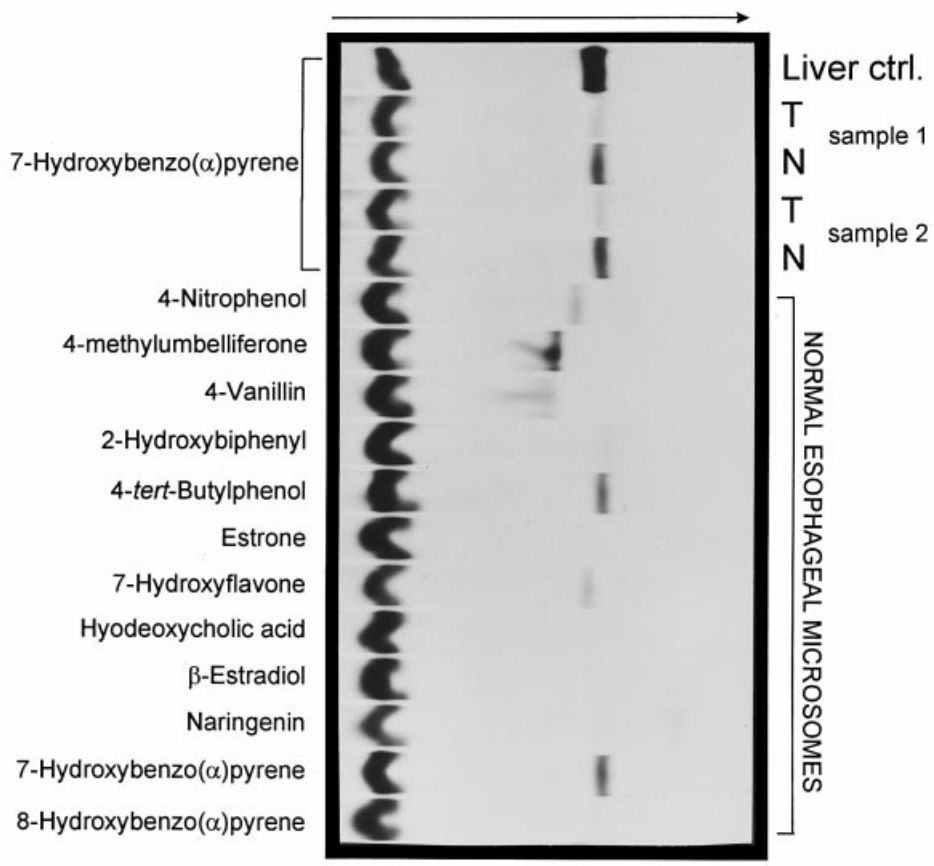

Figure 3 Specific UGT activities in squamous-cell carcinoma and normal oesophageal mucosa

Autoradiography of a TLC separation, indicating ^{14}C-labelled glucuronides (arrow shows the direction of separation). 7-Hydroxybenzo(α)pyrene glucuronidation was assayed in 25 μg of microsomal protein from carcinoma (T) and normal (N) tissue of samples 1 and 2, demonstrating significant downregulation of catalytic activity in oesophageal carcinoma. Liver microsomes (25 μg) were assayed as a control (ctrl.). In the lower part of the panel, specific UGT activities are demonstrated for 12 substrates using 25 μg of sample 2 normal-mucosa microsomal protein.

Table 1 Specific UGT activities in normal human oesophagus (normal sample 2)

All assays were performed in duplicate. Results did not vary by more than 5% in each assay.

Substrate	Specific activity (pmol/mg of protein per min)
4-Nitrophenol	70
4-Methylumbelliferone	420
Vanillin	152
2-Hydroxybiphenyl	17
4-*tert*-Butylphenol	149
Oestrone	10
7-Hydroxyflavone	50
Hyodeoxycholic acid	8
β-Oestradiol	14
Naringenin	31
7-Hydroxybenzo(α)pyrene	196
8-Hydroxybenzo(α)pyrene	17

Table 2 Glucuronidation of 7-hydroxybenzo(α)pyrene in oesophageal carcinoma and normal tissue

The substrate was 7-hydroxybenzo(α)pyrene in each case. All assays were performed in duplicate. Results did not vary by more than 5% in each assay.

Sample	Specific activity (pmol/mg of protein per min)
Liver control	1361
Sample 1, tumour	34
Sample 1, normal	166
Sample 2, tumour	33
Sample 2, normal	177

glucuronidation (Table 1). Bile acids and steroids have been characterized as the most prominent substrates for UGT2B proteins. These activity results are in agreement with the low levels of UGT2B mRNA found by DRT-PCR (Figure 2) and establish UGT1A7–10 as the dominant UGT proteins in human oesophagus.

UGT activity assays were further used to determine functional differences between carcinoma and normal oesophageal tissue. The hydroxylated metabolite of benzo(α)pyrene, 7-hydroxybenzo(α)pyrene, was used to compare glucuronidation in carcinoma and normal tissue (Figure 3 and Table 2). In both sample pairs 7-hydroxybenzo(α)pyrene glucuronidation was reduced five-fold in oesophageal squamous-cell carcinoma when compared with surrounding normal oesophageal mucosa (Table

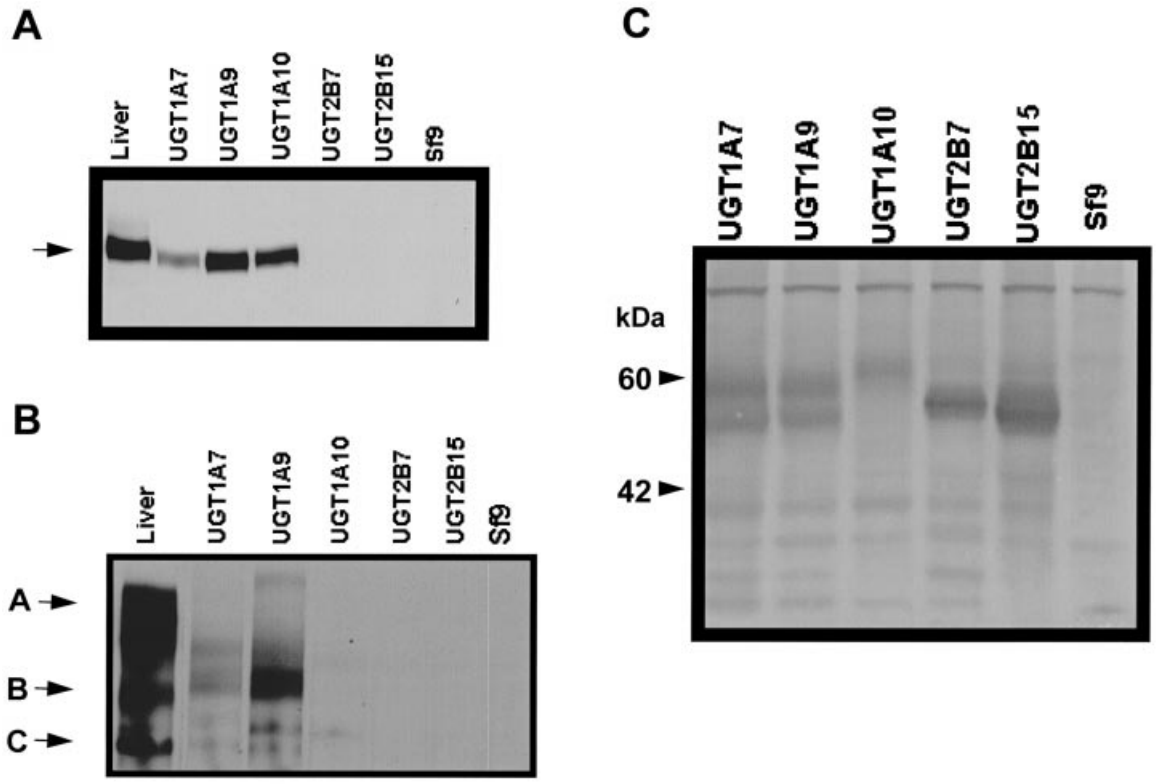

Figure 4 Analysis of 7-hydroxybenzo(α)pyrene and N-hydroxy-PhIP glucuronidation of recombinant UGT1A7, UGT1A10, UGT2B7 and UGT2B15

(**A**) Autoradiography of a TLC separation showing ^{14}C-labelled 7-hydroxybenzo(α)pyrene glucuronides catalysed by baculovirus-generated recombinant UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15. The exposure time for this film was 18 h. The specific activities of UGT1A7, UGT1A9 and UGT1A10 are shown in Table 3. (**B**) Autoradiography of a TLC separation showing ^{14}C-labelled N-hydroxy-PhIP glucuronides generated by human liver microsomes as well as UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15. The arrows indicate the different patterns of PhIP glucuronides generated with liver microsomes. These specific activities are shown in Table 3. The exposure time for this film was 9 days. (**C**) Western-blot analysis demonstrating the presence of all five recombinant proteins. Sf9 cells, *Spodoptera frugiperda* insect cells not expressing recombinant protein; molecular-mass markers, glutamate dehydrogenase (69 kDa) and carbonic anhydrase (42 kDa).

2). This finding indicates that 7-hydroxybenzo(α)pyrene glucuronidation may be performed by individual UGT proteins, which are differentially regulated between normal and carcinoma tissue. To examine this possibility, the individual UGT cDNAs were expressed in insect Sf9 cells for determination of catalytic activity.

Catalytic activity of recombinant UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15 using 7-hydroxybenzo(α)pyrene and N-hydroxy-PhIP

In a first experiment, recombinant UGT1A7, UGT1A9, UGT1A10, UGT2B7 and UGT2B15 proteins were expressed in baculovirus and used to determine their individual contribution to 7-hydroxybenzo(α)pyrene glucuronidation (Figure 4). The presence of all five recombinant proteins in Sf9 cells was documented by Western blot (Figure 4C). Catalytic analysis demonstrated that UGT1A9 was the most active towards 7-hydroxybenzo(α)pyrene, followed by UGT1A10 and then UGT1A7 (Table 3). Catalytic activity exhibited by UGT1A7 was approximately five-fold lower than UGT1A9. Neither UGT2B7 nor UGT2B15 catalysed the formation of 7-hydroxybenzo(α)pyrene glucuronides. Previous analyses have indicated that UGT2B10 is not active towards any of the conventional substrates used for glucuronidation analysis [8]. Experiments in our laboratory have also indicated that UGT1A8 is not active [18]; however, Mojarrabi and Mackenzie [19] have recently cloned a variant of UGT1A8 that is active and which displays minor activity towards 7-hydroxybenzo(α)pyrene.

Table 3 Glucuronidation of 7-hydroxybenzo(α)pyrene and N-hydroxy-PhIP by human liver and recombinant UGTs

All assays were conducted in duplicate on three different occasions. For these experiments, incubations were conducted for 2 h in the presence of 0.2 μCi [^{14}C]UDP-glucuronic acid. The distribution of the glucuronides as generated with human liver microsomes and displayed on the TLC after exposure to X-ray film were scraped off and quantified by liquid scintillation counting. For these experiments, the entire region of the TLC represented by the glucuronides was included. For example, the multiple N-hydroxy-PhIP glucuronide products (arrows A, B and C) shown in Figure 4(B) were combined in the analysis of specific activity in this Table. nd, not determined.

	Specific activity (pmol/mg of protein per min)	
Sample	7-Hydroxybenzo(α)pyrene	N-hydroxy-PhIP
Human liver	1343 ± 55	234 ± 10
UGT1A7	147 ± 7	3.00 ± 0.15
UGT1A9	869 ± 42	11.15 ± 0.53
UGT1A10	437 ± 19	2.73 ± 0.12
UGT2B7	11 ± 0.55	nd
UGT2B15	nd	nd

In summary, this experiment identifies UGT1A7, UGT1A9 and UGT1A10 as the dominant isoforms responsible for 7-hydroxybenzo(α)pyrene glucuronidation in human oesophagus.

In order to address the question of whether different carcinogens are glucuronidated by a different set of UGTs and thereby contribute specifically to the carcinogenesis in a specialized tissue, the environmental carcinogen metabolite N-hydroxy-PhIP was additionally analysed (Figure 4B). The TLC profile of N-hydroxy-PhIP glucuronidation is demonstrated following incubation of the substrate with liver microsomes. At least three prominent glucuronide products are present with liver microsomes (oesophagus tissue was not available for this experiment). It is known that 2-amino-1-methyl-6-phenylimidazo-(4,5-β)-pyridine (PhIP) can be glucuronidated at the N^3 imidazole-ring nitrogen [37], and control experiments using PhIP (results not shown) demonstrate that this glucuronide migrates to the position indicated by the top arrow (Figure 4B, marked A). Thus, the N-hydroxylated glucuronide would migrate to either the B or C positions. Figure 4(B) demonstrates that N-hydroxy-PhIP glucuronides are made with UGT1A7, UGT1A9 and UGT1A10. The use of equal amounts of expressed UGT1A7 and UGT1A9, as demonstrated by Western-blot analysis, confirms that UGT1A9 is the most active in the presence of N-hydroxy-PhIP (Table 3). UGT1A7 and UGT1A9 produce ^{14}C-labelled glucuronides that migrate with comparable R_F values, whereas the UGT1A10 glucuronides are characterized by a slower migration. None of the expressed UGTs produce the faster-migrating metabolite (arrow A). Because of the differing R_F values, this result indicates that UGT1A9 and UGT1A10 produce PhIP-glucuronides that must be conjugated at different positions. Clearly, additional UGTs in liver tissue are responsible for generating the faster-migrating PhIP glucuronides. In addition, we have demonstrated that UGT1A10 is not expressed in liver, thus the slowest-migrating metabolite (arrow C) seen with liver microsomes is most likely to have been generated by a form of UGT not expressed in oesophagus. Possibilities could include UGT1A1, UGT1A3, UGT1A4 or UGT2B4. Combined, the analyses of 7-hydroxybenzo(α)pyrene and N-hydroxy-PhIP glucuronidation have demonstrated that individual carcinogens are metabolized by specific UGT isoforms and that the tissue-specific expression of individual UGTs (as documented in Figures 1 and 2) in turn defines the carcinogen metabolism of a specific tissue.

DISCUSSION

Glucuronidation is a key process in human metabolic catabolism [1,3]. The superfamily of *UGT* gene products catalyses the formation of polar glucuronides from lipophilic substrates and thereby contributes to their inactivation, detoxification and ultimately elimination from the body. Although the majority of human UGTs have been identified in hepatocytes it has been shown that the human *UGT1A* locus is regulated in a tissue-specific fashion, leading to the existence of strictly extrahepatic UGTs, as in the case of UGT1A7, UGT1A8 and UGT1A10 [18]. Extrahepatic glucuronidation is therefore implicated in playing a significant and specialized role in drug metabolism, detoxification and cytoprotection.

The ability of UGT proteins to glucuronidate carcinogenic compounds, including primary amines and benzo(α)pyrenes, has led to the hypothesis that UGTs can impact the risk of chemical carcinogenesis in a tissue [16,27–30]. In this process UGTs may serve the dual function of eliminating potential carcinogens directly by glucuronidation or, as phase-II metabolic enzymes, by preventing the production of reactive oxygen species through elimination of hydroxylated carcinogens. In some situations, activated carcinogens are the end product of phase-I metabolism performed by cytochrome *P*-450 proteins. Xenobiotic glucuronidation can be expected to be of significance in tissues, such as the human oesophagus, with primary or prolonged contact to foreign material containing carcinogenic compounds. Epidemiology has linked the development of squamous-cell carcinoma of the oesophagus to tobacco-smoke consumption and ethanol intake [31–33,38]. It is believed that ethanol induces cytochrome *P*-450 enzymes such as CYP1A1 in oesophageal tissue [39,40] and increases bioactivation, leading potentially to an increase in carcinogen activation, e.g. of benzo(α)pyrenes or PhIP present in tobacco smoke. Studies have also documented a protective effect of fruit and vegetable consumption, which may be related to antioxidative compounds [41]. The expression, regulation and function of different UGT proteins in human oesophagus may therefore be an important factor in oesophageal physiology and pathophysiology.

In a first line of experiments we defined the expression of UGT1A and UGT2B mRNAs in oesophageal tissue. For this study, two paired samples of squamous-cell carcinoma/normal oesophageal mucosa were utilized. Qualified paired specimens are only obtained with considerable difficulties, for two medical reasons: (i) the majority of patients with oesophageal cancer receive radiation therapy (or a combination of radiation therapy and chemotherapy) prior to surgery, which ultimately renders the prospective resection specimen unsuitable for expression studies; and (ii) surgery in patients with very small tumours does not permit the removal of material from the resection specimen to preserve pathological diagnostic significance. The use of two sample pairs of this very rare material precludes a statistical analysis. However, the present study represents the first analysis of UGT1A and UGT2B expression in oesophagus and oesophageal cancer and the sample size does not reduce the significance of the novel findings obtained.

DRT-PCR established that oesophageal mucosa was characterized by the exclusive expression of UGT1A7, UGT1A8, UGT1A9 and UGT1A10. Except for UGT1A9, these UGTs are strictly extrahepatic proteins, which are also found in human gastric, biliary and colon tissue [16–18]. The expression pattern found in oesophagus is the first example of an exclusive expression of the entire *UGT1A7–10* gene cluster which has been suggested to have evolved to perform extrahepatic glucuronidation [18]. Expression of UGT1A7–10 may indicate a highly specialized glucuronidation in oesophagus. It provides the unique opportunity to study the function of these extrahepatic proteins without the co-expression of UGT1A1–6, which are present to varying degrees in most other tissues of the gastrointestinal tract [16–18]. UGT2B expression was limited to UGT2B7 and UGT2B10 in both samples, and UGT2B15 in one sample. Overall, UGT2B mRNA levels appeared to be substantially lower than UGT1A mRNA levels. The direct comparison of squamous-cell carcinoma with surrounding normal tissue collected from the same sample additionally showed that UGT2B mRNA was not differentially regulated. In contrast, UGT1A7 and UGT1A10 mRNAs exhibited differential regulation that was not observed for UGT1A8 or UGT1A9. The mRNA analysis suggested that in squamous-cell carcinoma of the oesophagus individual regulation of UGT isoforms is preserved and that only UGT1A7 and, to a lesser degree, UGT1A10 are differentially downregulated.

The functional implications of the demonstrated UGT1A regulation were further investigated by UGT catalytic activity assays. Specific UGT activities in normal oesophageal microsomes using a panel of 12 substrates, including phenolics, steroids, flavones and carcinogens, demonstrated the highest activities

with phenolic xenobiotics, which have been shown to be classical UGT1A substrates. This suggests a predominance of UGT1A protein activity in oesophageal microsomes and is in agreement with the low-UGT2B mRNA levels demonstrated by DRT-PCR. The benzo(α)pyrene metabolite 7-hydroxybenzo(α)pyrene was used as a substrate to determine differences of glucuronidation rates between oesophageal carcinoma and normal tissue. This experiment showed a five-fold downregulation of glucuronidation rates.

To further characterize the contribution of individual UGT proteins expressed in oesophageal microsomes, recombinant protein was utilized. Previous analyses with active UGT1A8 demonstrated limited activity toward the glucuronidation of 7-hydroxybenzo(α)pyrene [19]. Published data also indicate that UGT2B10 is not an active protein [8]. UGT1A9, UGT1A7, UGT1A10, UGT2B7 and UGT2B15 were expressed and tested for 7-hydroxybenzo(α)pyrene glucuronidation, and additionally for N-hydroxy-PhIP. UGT1A9 was identified as the most active 7-hydroxybenzo(α)pyrene transferase. As shown previously [18], UGT1A7 and UGT1A10 were also active in the presence of 7-hydroxybenzo(α)pyrene. The UGT2B proteins did not catalyse 7-hydroxybenzo(α)pyrene glucuronidation. Although UGT1A9 is expressed in oesophagus and recombinant protein is found to be catalytically active towards 7-hydroxybenzo(α)pyrene, this isoform is not differentially regulated between normal and carcinoma tissues. Therefore, since UGT1A7 and UGT1A10 transcripts are shown to be down-regulated, the reduction in 7-hydroxybenzo(α)pyrene glucuronidation in oesophageal carcinoma may be attributed to the regulation of these UGTs.

The ability of the oesophageally expressed UGT proteins to glucuronidate PhIP was also analysed, since this substrate has been implicated as a potential dietary carcinogen. PhIP undergoes N-hydroxylation by cytochromes P4501A1 and P4501A2 at the two-amino position [42] to generate the mutagenic metabolite [43]. Since P4501A1 is not expressed abundantly in liver [44] and P4501A2 is limited to expression in hepatic tissue, it has been proposed [45] that N-hydroxy-PhIP is transported from the liver in the circulation to target tissues where it forms DNA adducts. Thus, understanding which of the UGTs participates in the metabolism of PhIP is important. Having determined the pattern of *UGT* gene expression in oesophageal tissue, an accurate estimation of those UGTs that may serve to metabolize PhIP can be made. Analysis of N-hydroxy-PhIP glucuronidation demonstrated that UGT1A9 was most active towards this substrate. UGT1A7 and UGT1A10 demonstrated comparable activities, although the UGT1A10-generated metabolite was different from that generated by UGT1A7 and UGT1A9, as determined by the relative R_F values of the glucuronidated substrate. Liver microsomes generate three different PhIP glucuronide metabolites, two of which correspond to those generated by UGT1A7, UGT1A9 and UGT1A10. Interestingly, only UGT1A9 is expressed in liver, indicating that additional forms of the transferase contribute to the overall glucuronidation rate of PhIP in liver.

In combination, these data are significant for two reasons: (i) they demonstrate that there exists a substrate specificity for extrahepatic UGTs towards different environmental carcinogen metabolites; and (ii) the substrate specificity combined with the differential regulation of UGT expression in individual tissues may be the biochemical basis of differences in carcinogen glucuronidation in specific human organs. These results suggest that differential UGT expression can define individual risk factors of cytotoxicity and genotoxicity in a specific tissue. This implicates the hypothesis that UGTs may contribute to a mucosal line of defence in human tissues with contact to external matter of surface organs such as the gastrointestinal tract. An additional factor in this process may be the differential regulation of UGT1A7 in squamous-cell carcinoma of the oesophagus, which is likely to contribute to the decrease of 7-hydroxybenzo(α)pyrene glucuronidation activity observed in the cancer microsomes. This example, using 7-hydroxybenzo(α)pyrene as a substrate and the comparison with N-hydroxy-PhIP glucuronidation, establishes that the regulation of individual *UGT1A* genes and possibly of *UGT2B* gene products may affect the ability to glucuronidate and detoxify individual substrates.

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