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• ORIGINAL RESEARCH •

Cloning of UGT1A9 cDNA from liver tissues and its expression in CHL cells

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Abstract

AIM: **To clone the cDNA of UGT1A9 from a Chinese human liver and establish the Chinese hamster lung (CHL) cell line expressing human UGT1A9.**

METHODS: **cDNA of UGT1A9 was transcripted from mRNA by reverse transcriptase-ploymerase chain reaction, and was cloned into the pGEM-T vector which was amplified in the host bacteric *E. Coli* DH5α. The inserted fragment, verified by DNA sequencing, was subcloned into the *Hind* III/*Not* I site of a mammalian expression vector pREP9 to construct the plasmid termed pREP9-UGT1A9. CHL cells were transfected with the resultant recombinants, pREP9-UGT1A9, and selected by G418 (400 mg·L^{-1}) for one mo nth. The surviving clone (CHL-UGT1A9) was harvested as a pool and sub-cultured in medium containing G418 to obtain samples for UGT1A9 assays. The enzyme activity of CHL-UGT1A9 towards propranolol in S9 protein of the cell was determined by HPLC.**

RESULTS: **The sequence of the cDNA segment cloned, which was 1666 bp in length, was identical to that released by Gene Bank (GenBank accession number: AF056188) in coding region. The recombinant constructed, pREP9-UGT1A9, contains the entire coding region, along with 18 bp of the 5′ and 55 bp of the 3′ untranslated region of the UGT1A9 cDNA, respectively. The cell lines established expressed the pro tein of UGT1A9, and the enzyme activity towards propranolol in S9 protein was found to be 101±24 pmol·min^{-1}·mg^{-1} protein (n = 3), but was *Not* detectable in parental CHL cells.**

CONCLUSION: **The cDNA of UGT1A9 was successfully cloned from a Chinese human liver and transfected into CHL cells. The CHL-UGT1A9 cell lines established efficiently expressed the protein of UGT1A9 for the further enzyme study of drug glucuronidation.**

Subject headings UGT1A9; cloning; glucuronidation; cell lines

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INTRODUCTION

Most organisms are exposed to a range of lipophilic compounds and converted them into excretable hydrophilic compounds. This metabolism of foreign compounds (xenobiotics) can be divided into two phases. For phase I metabolism, a reactive group is mostly introduced into the xenobiotic molecule. These reactions are mainly catalyzed by the cytochrome P450 monooxygenase system which consists of cytochrome P450s (CYPs) and cytochrome P450 reductase (CPR). For phase II metabolism, the reactive metabolite is conjugated to small, hydrophilic endogenous molecules such as glucuronic acid. The conjugation of this cofactor to xenobiotics is catalyzed by UDP-glucuronosyltransferases (UGTs). Since xenobiotic metabolizing enzymes have to catalyze the metabolism of structurally very divers e substrates, the various enzyme systems (e.g. CYPs and UGTs) comprise several isozymes that differ in their catalytic properties. The members of a given enzyme system have been grouped into families and subfamilies based on sequence homologies. In UGTs, two enzyme families termed UGT1 and UGT2 have been described.

The UGT1 locus is highly conserved between species[1]. UGT1A is a subfamily of UGT1 gene complex that is located at chromosome 2q37. UGT1A subfamily is encoded by tandem individual promoters and their first exons are linked by differential splicing to four common exons. As one of the isoforms, UGT1A9, is mainly expressed in liver. UGT1A9 can be induced by polycyclic aromatic hydrocarbons (PAHs), and therefore the drug glucuronidation catalyzed by UGT1A9 will be increased in cigarette smokers who inhale PAHs[2].

Human hepatic UDP-glucuronosyltransferases (UGT) is a family of microsomal enzymes that catalyze the glucuronidation of many important drugs, xenobiotics and endogenous compounds. Attempts to characterize the microsomal enzymes by conventional purification technique are often frustrated due to its instability. UGT isoenzyme expressed by cells is a useful tool for characterizing UGT's function. The cDNA cloning of UGTs from various sources (rabbit, rat, monkey, human beings, etc.) and their expression in cell lines were widely used for the gene characterization and function study of UGT isoforms[3-9]. In order to study tie drug metabolisms by UGTs, the cDNA encoding UGT1A9 was cloned from human liver and expressed in Chinese hamster lung (CHL) cell line in this study. The enzyme expressed was extracted and its activity was assayed with a substrate of propranolol which is a nonselective β-adrenergic blocking agent and can be used widely clinically[10].

MATERIALS AND METHODS

Isolation of RNA from human liver tissue

Human liver tissue was obtained from a surgical specimen of Chinese and stored at -80℃ until use. The total RNA was isolated with TRIzol reagent (Gibco Corp, USA)

UGT1A9 cDNA transcription

cDNA was transcr ipted from mRNA by revere transcriptase polymerase chain reaction (RT-PCR). Five μg of the total RNA and 2 μg of random primer (SANGON, Shanghai) in deionized water containing

DEPC (1 g·L^{-1}) were denatured at 65℃ for 15 min, then 4 μL 5 ×reverse transcriptase buffer, 3 μL10 mmol·L^{-1} dNTP, 1 μL M-MuLV reverse transcriptase (200 U) (Fermentas) and essential deionized water containing DEPC (1 g·L^{-1}) were added to have the total volume of 20 μL. The reaction was performed at 25℃ for 10 min, then 42℃ for 1 hour, and 70℃ for 10 min to inactivate the reverse transcriptase. The product was finally held at 4℃. Two μL of the reactant was mixed with 2 μL of 10 mmol·L^{-1} dNTP, 30 pmol of PCR primers and 3.5 U of DNA ploym erase (Perkin-Elmer Corp). The total volume of 100 μL was reached by adding deionized water. Two 26 meroligonucleotides as PCR primers were designed according to the DN A sequence of UGT1A9 (GenBank accession no. AF056188). The sense oligonucleo tides corresponding to base positions 1 to 26 was 5'-CTAAGCTTCAGTTCTCTGATGGCTTG-3 'with a restriction site of *Hind* III, and the anti-sense one, corresponding t o the bases position from 1641 to 1666, was 5'-GTTGGAAATG CCTAGGGAATGGTTC-3'. The poly merase chain reaction (PCR) was performed at 94℃ 2 min, then 94℃ 15 s, 60.1℃ 30 s and 72℃ 2 min for 31 cycles, and 72℃ for 10 min. The product was finally held at 4℃. An agrose gel electrophoresis was carried out with 10 μL of the PCR solution to check the 1666 bp DNA amplified.

Construction of recombinant pGEM-UGT1A9 and sequencing of UGT1A9

The PCR product of about 1.5 kb was isolated and ligated with pGEM-T (Promega) vector by T4 DNA ligase (Fermentas). *E. Coli* DH5α was transformed with the resulted recombinants pGEM-UGT1A9[11] and the positive bacteria colonies were screened by ampicillin resistant and blue-white screening with X-gal and IPTG. The cDNA of UGT1A9 subcloned in pGEM-T was sequenced on both strands by dideoxy chain-termination method marked with BigDye with primers of T7 and SP6 promoters and a specific primer of 5'-CAAGTATCGT GTTGTTCGC-3'. The termination products were resolved and detected using an automated DNA sequencer (Perkin-Elmer-ABI Prism 310, Foster City, CA).

Construction of the pREP9 based expression plasmid for UGT1A9

The *Hind* III-*Not* I fragment of the human UGT1A9 cDNA cleaved from the selected and amplified recombinant pGEM-UGT1A9 by *Hind* III and *Not* I digestion was purified by agarose electrophoresis and cloned directly into a unique *Hind* III-*Not* I site within the multicloning site of the mammalian expression vector pREP9 (Invitrogen, San Diego, CA) with T4 ligase.

Transfection and selection

Chinese hamster lung (CHL) cells were transfected with the resultant recombinants, pREP9-UGT1A9, using a calcium phosphate method[12]. After 24 h incubation at 37℃, the culture was rinsed and re-fed with fresh growth medium. Seventy-two h after transfection, the culture was split and then selected in the culture medium containing the neomycin analogue G418 (Gibco BRL, MD) (400 mg ·L^{-1}). The selective medium was changed every 3-4 d to remove dead cells and allow the growth of resistant colonies. After 1 mon, surviving clonies (termed C HL-UGT1A9) were harvested as a pool and propagated in medium containing G41 8.

Preparation of S9 of CHL-UGT1A9

CHL-UGT1A9 cells grown in the culture medium containing G418 (400 mg·L^{-1}) were rinsed with phosphate balanced solution (PBS), scraped and collected from the bottle with 11.5 g·L^{-1} KCl in aqua solution and t hen sonicated 3 s for 5 times with 5 s of interval break. The resulted homogenate was centrifuged at 9000×*g* for 20 min and the supernatant (S9) was transferred carefully to a clean tube for assay or storage under -70℃. The protein in S9 was determined by the same method that was used in our previous paper[13].

UGT assay

The UGT1A9 activities of S9 fraction were determined by the gl ucuronidation of propranolol. The assay was performed in a total volume of 100 μl containing final concentrations of 0.2 mmol·L^{-1} propranolol, 1 mmol · L^{-1} UDPGA, 1g ·L^{-1} Triton X-100, 50 μg of S9 protein in 50 mmol· L^{-1} Tris-HCl, 10 mmol·L^{-1} $MgCl_2$ buffer, pH 7.8 at 37℃. The mixtures were pre-incubated and the glucuronidation was started by the addition of UDPGA and stopped after 2 h by the addition of 100 μL of methanol. The mixtures were stirred thoroughly and centrifuged at 10 000 r·min^{-1} for 10 min. Un-reacted propranolol in the layer of reactant was d etermined by HPLC and the enzyme activity was calculated according to the amount of propranolol declined after incubation.

HPLC analysis of propranolol metabolized by S9 of CHL-UGT1A9

The concentration of propranolol metabolized by S9 of CHL-UGT1A9 was assayed by the HPLC procedure[13] with modification to the mobile phase. Twenty mL of the sample was applied to a reversed phase column (Shim-pack CLC-ODS15 cm×0.6 cm id, 10 μm particle size). Propranolol was monitored with a UV detector at 290 nm. The mobile phase is made up with ammonium acetate buffer (4.0 g ammonium acetate, 10 mL acetate acid and de-ionized water in 1 L)-methanol-acetonitrile (2:1:1), and to 500 mL mobile phase add 0.7 mL triethylamine as the elution modifier. The flow rate is 1.0 mL·min^{-1}.

RESULTS

Construction of recombinants

The recombinant of pGEM-UGT1A9 (Figure 1) was const ructed with the human UGT1A9 inserted into the cloning site of vector pGEM- T between the promoters of T7 and SP6. Selection and identification of the recombinant was carried out by *Hind* III / *Not* I endonuclease digestion and agarose elector phoresis (Figure 3). The cloned DNA segments in selected recombinants were sequenced completely. According to the results of DNA sequencing, the cDNA in a selected recombinant was identical to the DNA sequence of UGT1A9 reported by Ciotti-M *et al* (GenBank accession no. AF056188) in the reading frame. The restriction sites of *Hind* III and *Not* I in the recombinant were used for the subcloning of insertion fragment into an expression vector.

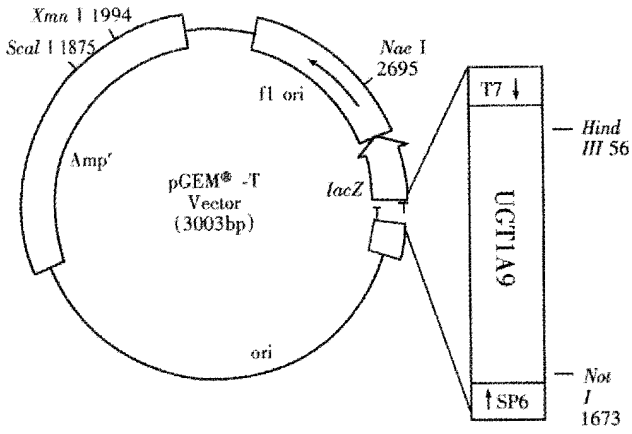

Figure 1 Scheme of pGEM-UGT1A9.

The *Hind* III/*Not* I fragment (1.5 kb) containing the complete *UGT*1A9 cDNA was subcloned into the *Hind* III/*Not* I site of mammalian expression vector pRE P9 (Figure 2). Selection and identification of the recombinants were carried out by *Hind* III/*Not* I endonuclease digestion and agarose electrophoresis (Figure 3). The re sulting plasmid was designated as pREP9-*UGT*1A9 which contained the entire coding region, along with 18 bp of the 5' and 55 bp of the 3' untranslated region of the *UGT*1A9 cDNA, respectively. In addition, the neo gene of the plasmid confers the G418 resistant phe*Not*ype to CHL cells for the selection of transfected cells.

Establishment of recombinant cell lines with UGT1A9 enzyme activity

CHL cells were transfected with pREP9-UGT1A9, and selected with G418 (400 mg·L^{-1}). The surviving clone was propagated and the cell line termed CHL-UGT1A9 was established. The preparation S9 was prepare d from CHL-UGT1A9 cells harvested for UGT1A9 activity assay by HPLC. Figure 4 shows t he typical elution of propranolol in incubation solution. The UGT enzyme activity towards propranolol in S9 protein was found to be 101±24 pmol·min^{-1}·mg^{-1} (n = 3), but was *Not* detectable in parental CHL cells.

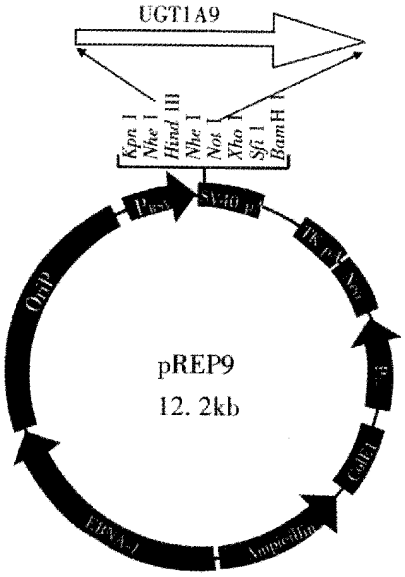

Figure 2 Scheme of pREP9-UGT1A9.

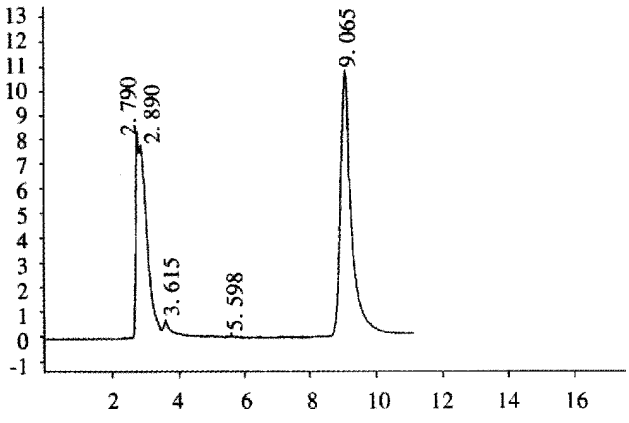

Figure 3 Electrophoresis identification of recombinants constr ucted.

Lanes 1: Marker (λ / EcoR I and *Hind* III); 2: PCR product of *UGT*1A9 from Chinese human liver; 3: recombinant of pGEM-UGT1A9; 4: recombi nant of pGEM-*UGT*1A9 cleaved by *Hind* III and *Not* I; 5: recombinant o f pREP9-*UGT*1A9; 6: recombinant of pREP9-*UGT*1A9 cleaved by *Hind* III and *Not* I; 7:pREP9 expression vector; 8:Marker (λ / *Hind* III).

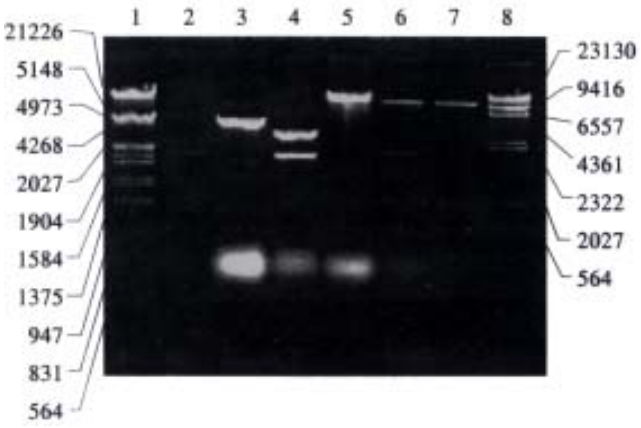

Figure 4 Chromatogram of propranolol after incubation with S9 prepared from CHL-UGT1A9 cell. A Shi m-pack CLC-ODS column (15 cm×0.6 cm i.d.) was used. The mobile phase was constituted with ammonium acetate buffer-methanol-acetonitrile (2:2:1) and 1.4mL·L^{-1} triethy lamine with the flow rate at 1.0 mL·min^{-1}. Propranolol was monitored at 290 nm. propranolol: t_R = 9.065 min.

DISCUSSION

UGTs are involved in the conjugation of UDP-glucuronic acid (UDPGA) to a variety of chemicals, drugs, and endogenous compounds. The elimination of hydrophobic chemicals from cells is aided by their conversion to water-soluble glucuronides. UGTs are closely relatied to the system of cytochrome P450 monooxygenase, and involved in the transportation of carrier and the passage of drugs through cell phospholipid bilayer. In most cases, the lipophilic compounds are converted by phase I metabolism to the substrate f or glucuronidation by obtaining an essential function (such as carbon, nitrogen, sulfur and oxygen), but in many cases xenobiotics and endogenous substances canalso be glucuronidated by UGTs without the phase I metabolism. The xenobiotic metabolizing cytochrome P450 monooxygenase system and the UGTs reside mainly in the endoplasmic reticulum. However, CYPs and the CPR are localized on the cytosolic side of the endoplasmic reticulum, which the UGTs are localized on its luminal side[14]. UGTs are latent enzymes, needing activation (in general by detergents) to express its maximal activity.

Numerous reports revealed that glucuronidation is a major pathway involved in the metabolism of drugs, exogeno us, and numerous endogenous compounds such as bile acids and steroid hormones. E ach UGTs family or subfamily has its own substrates but the substrate spectrum a re partly overlapped. UGT1 has substrates such as thyroid hormone[15], SN-38[16], bilirubin[17,18], opioids, bile acids, fatty acids, retinoids, c iprofibrate , furosemide, dilunisa, catechol estrogens, coumarins, flavonoids, anthraquinone s, EM-652 (an active antiestrogen)[19] and phenolic compounds[20]. UGT2 catalyzes substrates such as estrogens, androgens, morphine, AZT, and retinoicacid, epirubicin[16,21,22,23], etc. UGT1A9 is a member of UGT1A subfamily. The endogenous substrates for UGT1A9 are estrone, 4-hydroxyestrone, ethinylestradiol, retinoic acids, etc, and exogenous substrates include propofol, propranolol, paracetamol, S-naproxen, ketoprofen, ibuprofen, entacapone, some mutagenic arylamines, etc[2,24-26]. UGT1A9 was found to have regioselectivity on the glucuronidation of hydroxyl group of carbohydrate-containing drugs[27].

UGTs are expressed extensively in organs and tissues, and they may play a key role in the regulation of the level and action of steroid hormones in steroid target tissues. Organs that express UGTs include liver, kidney, gastrointestinal tract[28-29], olfactory[30], jejunum,

ileum[31], prostate[32-33], colon[34]. UGT1A9 is mainly expressed in liver, and also expressed in steroid targets[35] and colon[34].

UGTs are inducible enzymes. In most cases this induction is due to increased transcription of the corresponding genes but sometimes it is also due to an improved stability of proteins. The pattern of enzymes affected is dependent on the inducing agent. Usually, phenobarbital induces mainly enzymes within UGT2 family, and methylcholanthrene induces enzymes belonging to the UGT1 family[28]. Other chemicals that induce UGTs include aryl hydrocarbon receptor ligands or oltip raz[36], flavonoid chrysin[37], and t-butylhydroquinone and 2,3,7,8-tetrachloro dibenzo-p-dioxin[38], etc. UGT1A9 can be induced by polycyclic aromatic hydro carbons (PAHs)[39]. On the other hand, UGTs can also be inhibited, for example by uridine diphosphate[40], and N-glycosylation is involved in the functional properties of UDP-glucuronosyltransferase enzymes[41].

To clone and express UGTs in cells can help screen substrates that an isoenz yme is responsible. The prod uction of a UGT enzyme protein using transgenic cell lines is a practical manner to study its function[42-43]. We report here the cloning of UGT1A9 cDNA and establishment of a CHL cell line expressing UGT1A9 from a Chinese human liver. The full-length cDNA, UGT1A9, that encodes for a human UDP-glucuronosyltransf erase protein, was isolated from a Chinese human liver total RNA. To achieve high expression levels of UGT1A9, the UGT1A9 cDNA was cloned into the eukaryotic expressi on vector pREP9, which we had previously used in this laboratory for the express ion of human CYP450 1A1, 2B6, 3A4, etc in CHL cells[44-45]. The salient feature of this vector has an EBV origin of replication and nuclear antigen (EBNA-1) to allow high-copy episomal replication in mammal cell lines. The Rous sarcoma virus long terminal repeat (RSV LTR) early promoter controls the expression of the U GT1A9 cDNA. As *Not*ed under "Results", the isolated clone contains a 1592-nuc leoti de open reading frame flanked by 18 and 55 base pairs of 5' and 3' noncoding sequences, respectively. The DNA sequence in the reading code frame is identical to that reported (GenBank accession no. AF056188). The expression of a protein that catalyzed the glucuronidation of propranolol was proven in the Chinese hamster lung cells transfected with the recombinant plasmid pREP9-*UGT*1A9.

Conjugation with glucuronic acid is an important biotransformation pathway for a large number of clinically used drugs. In human intestinal, UGTs play an important role in the detoxification of xenobiotics compounds and, in some cases, may limit the bioavailability of therapeutic agents[20]. The deficient of a UGTs isoenzyme, may cause disease and clinical incident[46-47], the typical example was serious adverse events associated with chloramphenicol toxicity in neonates. Human UGTs are regulated in cases of healthy condition and exposure of harmful environmenta l carcinogens[48-50]. Moreover, UGT was identified as an antigenic tar get in a subgroup of liver- kidney microsomal auto-antibodies[51]. Hence, it is very necessary to undertake the study of functions and characteristics of UGTs. Over the last decade, some research papers were published about the usage of cloned and expressed human UGTs for the assessment of human drug conjugations and identification potential drug interactions[6-8]. However, the information gap still exists regarding the enzymatic aspects of UGTs to drugs elimination and its potenti al impact on therapy. More researches on the drug metabolism by UGTs are necessary for effective translation of scientific information into clinically applicable knowledge. As has been shown with the CYPs, coupling of basic and clinical science is needed to continually improve our understanding of the UGTs. Many factors are known to influence the activities of UGTs involved in drug metabolism, hence plasma clearances of glucuronidated drugs. Such factors include age (especially neonatal period), cigarette smoking, diet, certain disease states, drug therapy, ethnicity, genetics and hormonal effects. Knowledge of the profile, substrate specificities and regulation of human UGTs remains limited and cons equently it is still generally *Not* possible to predict the effects of specifice nvironmental and genetic factors on the metabolism and pharmacokinetics of individual glucuronidated drugs. Future investigations must define the substrate spec ificities of the various UGTs and investigate mechanisms by which the separate isozymes are regulated. Only then will it become possible to rationalize (and predict) the alterations in pharmacokinetics and response to glucuronidated drugs in specific patient groups.

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