IDENTIFICATION AND CHARACTERIZATION OF A FUNCTIONAL TATA BOX POLYMORPHISM OF THE \textit{UDP GLUCURONOSYLTRANSFERASE 1A7} GENE

by

Tim O. Lankisch, Arndt Vogel, Stefan Eilermann, Anette Fiebeler\textsuperscript{1}, Britta Krone, Ayse Barut, Michael P. Manns, and Christian P. Strassburg

Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany, and \textsuperscript{1}Department of Medicine of the Charite, Franz Volhard Clinic, HELIOS Klinikum-Berlin, Berlin, Germany
Abstract:

UDP-glucuronosyltransferases (UGT) detoxify bilirubin and therapeutic drugs, a process influenced by single nucleotide polymorphisms (SNPs) in their structural genes and promoter elements. UGT1A1*28 is a functional UGT promoter polymorphism associated with Gilbert’s disease and severe irinotecan toxicity, which also occurs in the absence of UGT1A1*28. The aim of this study was to identify and characterize UGT promoter variants relevant for irinotecan detoxification. Recombinant UGT1A proteins were analyzed for irinotecan metabolite glucuronidation by UGT activity assays. In 427 healthy blood donors and 71 homozygous UGT1A1*28 carriers the 5’-untranslated region of the UGT1A7 gene locus was studied. A SNP was detected by allelic discrimination and characterized by reporter gene experiments. A novel –57 T>G SNP with a gene frequency of 0.39 in healthy blood donors was identified in the putative TATA box of the UGT1A7 gene reducing promoter activity to 30%. It is in linkage dysequilibrium with a variant of the UGT1A7 first exon present in the reduced activity UGT1A7*3 and UGT1A7*4 alleles. Homozygous UGT1A1*28 carriers simultaneously carried this variant in 98%. We identified a novel reduced function TATA box SNP of the UGT1A7 gene which catalyzes irinotecan metabolite detoxification. Its association with variants of the UGT1A1 promoter and UGT1A7 gene may influence irinotecan metabolism. Our finding emphasizes the importance of combinations of structural and regulatory gene polymorphisms which may be useful as a marker of drug toxicity.
The UDP-glucuronosyltransferase family of enzymes is a central metabolic system for the glucuronidation of hydrophobic endobiotic and xenobiotic compounds (Tukey and Strassburg, 2000). Glucuronidation leads to the formation of water soluble metabolites for an array of compounds including steroid hormones, bilirubin and bile acids as well as a vast array of therapeutic drugs, environmental organic substances including known human mutagens (Tukey and Strassburg, 2001). Among the most relevant drugs which undergo glucuronidation are morphine (Coffman, et al., 1997), acetaminophen (Bock, et al., 1993), chloramphenicol (de Wildt, et al., 1999), transplant immunosuppressants such as cyclosporine A and tacrolimus (Strassburg, et al., 2001), but also the widely used anti-tumor drug metabolite of irinotecan SN-38 (Haaz, et al., 1997, Ciotti, et al., 1999). Alterations of glucuronidation activities in the individual are a mechanism by which interindividual profiles of drug metabolism are believed to impact drug efficacy, drug side effects and the predisposition towards environmental mutagen-associated diseases such as cancer (Tukey and Strassburg, 2001, Tukey, et al., 2002).

The human UGT1A proteins have been implicated as risk factors for both the development of cancer and unwanted drug side effects. This risk is determined by 3 differing features of the UGT1A gene locus. First, the UGT1A gene locus is regulated and expressed in a tissue specific fashion encompassing the hepatic isoforms UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 (Strassburg, et al., 1998, Strassburg, et al., 1997, Strassburg, et al., 1999). In extrahepatic tissues such as mouth, esophagus, intestine, pancreas and colon non-hepatic enzymes (UGT1A7, UGT1A8 and UGT1A10) have been detected conferring a tissue specific profile of glucuronidation to each organ which has been characterized by the analysis of tissue microsomes (Strassburg, et al., 1997, Strassburg, et al., 1999, Vogel, et al., 2002, Ockenga, et al., 2002). Second, the analysis of different tissues in the human gastrointestinal tract has shown that UGT1A and UGT2B genes are regulated in a polymorphic interindividual fashion leading to differing steady state levels of UGT mRNA, protein and enzymatic glucuronidation activity (Strassburg, et al., 1998, Strassburg, et al., 2000). The molecular basis of this feature is presently not completely understood. Third, an increasing number of single nucleotide polymorphisms (SNP) has been identified for all known UGT1A isoforms leading to catalytically altered UGT1A protein variants (Bosma, et al., 1993, Burchell, 2003, Ciotti, et al., 1997, Guillemette, et al., 2000, Strassburg, et al., 2002). Together, this opens the possibility for a considerable number of combinations which represent the biochemical basis...
of highly interindividual profiles of glucuronidation conserved during evolution (Ehmer, et al., 2004). These SNPs mostly lie within the coding regions of the UGT1A gene domains and only one SNP within the promotor region has been identified to date. UGT1A1*28 is characterized by the insertion of a TA into the A(TA)6TAA element leading to A(TA)7TAA and a reduction of promoter activity to 30% (Bosma, et al., 1995, Monaghan, et al., 1996). This SNP is the genetic basis of Gilbert-Meulengracht’s disease leading to unconjugated non-hemolytic hyperbilirubinemia because UGT1A1 is the only efficient metabolic pathway for the elimination of bilirubin from the human body (Strassburg and Manns, 2000). In addition other variants such as A(TA)5TAA and A(TA)8TAA have been described in ethnically distinct populations (Strassburg and Manns, 2000). However, apart from forming the genetic basis of this uncomplicated hepatic disease UGT1A1*28 carrier status has been linked to the susceptibility towards breast cancer (Guillemette, et al., 2000) and the risk of unwanted intestinal side effects as well as myelotoxicity in colorectal cancer patients treated with irinotecan (Ando, et al., 1998, Gagne, et al., 2002). Irinotecan and its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), which has a 100-1000 fold higher activity than irinotecan, undergo glucuronidation by UGT1A1 and other UGT (Ciotti, et al., 1999). However, the UGT1A1*28 polymorphism is not capable of explaining all cases of irinotecan-associated toxicity indicating the existence of additional markers (Innocenti, et al., 2004).

In the present study we identified a functional polymorphism of the promotor region of the human gastrointestinal UGT1A7 and provide evidence for an association with exon SNPs as well as the UGT1A1*28 promotor polymorphism implicating a possible role for irinotecan toxicity in humans.
Materials and Methods

Patients:

*Gilbert-Meulengracht disease*: Blood samples were collected from patients worked up for the presence of Gilbert-Meulengracht’s disease at the Department of Gastroenterology, Hepatology and Endocrinology of Hannover Medical School. In 200 patients (age: 0.4 to 71.3 years, average 17.2 years, 120 male, 80 female) with suspected Gilbert’s disease genotyping of the UGT1A1*28 promoter polymorphisms was performed using PCR, direct sequencing and temperature gradient electrophoresis as previously described (Strassburg, et al., 2002).

*Healthy blood donors*: Blood samples were obtained from 427 healthy blood donors from the Department of Transfusion medicine/Blood Bank of Hannover Medical School.

Genomic DNA. Genomic DNA was isolated from full blood samples by the NucleoSpin Blood XL Kit according to the recommendations of the manufacturer (Machery & Nagel, Dueren, Germany).

PCR analysis: The UGT1A7 promoter sequence was amplified by PCR. The forward primer (5’-GTACACGCTTCTTGGAGGCAG-3’) was located from base pair (bp) –103 to -80 upstream of the ATG start codon (Genbank accession number U39570), whereas the reverse primer (5’-TGCACTTCGCAATGGTGCCGTCCA-3’) was located from bp +292 to +315 downstream of the ATG start codon. Sequencing of both primer regions excluded allelic variants potentially affecting primer binding. The 371-bp product was amplified using the following protocol: hot start at 94°C for 5 min, followed by 32 cycles of 94°C for 30 sec, 63°C for 30 sec and a final 7 min elongation reaction at 72°C. PCR was run on a Perkin Elmer GeneAmp PCR 2400 system (Perkin Elmer, Juegesheim, Germany).

Sequence Analysis. Sequence PCR was performed using the Dye Terminator Cycle Sequencing Kit 1.1 (Applied Biosystems, Darmstadt, Germany). The nucleotide sequences were determined on an ABI 310 automated sequencer (Applied Biosystems).

Allelic discrimination genotyping: Approximately 10 ng of genomic DNA were used as a template in Taqman 5’-nuclease assays. Primers and Probes specific for each SNP were designed with Primer Express software (Applied Biosystems) and labelled with either 6-FAM or VIC as reporter dyes and MGB-NFQ (Applied Biosystems) as quencher (Table 1A). The
Taqman assays were performed using 600 nM primer concentrations and 200 nM probe concentrations (Applied Biosystems) and qPCR Mastermix Plus (Eurogentec, Seraing, Belgium). The run consisted of a hot start at 95°C for 10 minutes and 35 cycles of 94°C for 15 sec and 61°C for 1 min. All assays were performed in 25 µl reactions in 96-well trays using an ABI 7000 instrument (Applied Biosystems).

**Construction of UGT1A7 luciferase reporter gene vectors:** A 251 bp DNA fragment was amplified by PCR from two healthy blood donors exhibiting the −57T and the −57G variants. Positional cloning was performed using primers containing an Xho I (5′-ACCGCTCGAGCAGAGAACTTCAGCCCAGAGCC-3′, located between -22 to -1 upstream of the UGT1A7 coding region) and a Kpn 1 (5′-GGAGGTACCAGGGCATGATCTGTCCCCAAGG-3′, located between -251 to -228 upstream of the UGT1A7 coding region) restriction enzyme site and the fragments were inserted into the pGL3 basic vector using the fast link ligation Kit (Fermentas, St. Leon-Rot, Germany). Mutagenesis of the putative box element was performed using specific primers (Table 1B) and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The sequences of all inserts were confirmed by DNA sequencing using the pGL primer 2 rev (5′-CTTTATGTTTTTGGCGTCTTCC -3′).

**Luciferase Assays:** Human embryonic kidney (HEK) 293 cells, Hepatoma (Hep) G2 cells, human gastric cancer (Kato III) and alveolar epithelial cells (A549) cells were transfected with the pGL3 UGT1A7-TATA box vectors in addition to PhRL-TK plasmid (Dual-Reporter Assay, Promega, Mannheim, Germany) using Lipofectin (Invitrogen, Karlsruhe, Germany) following the manufacturer’s instructions prior to harvesting after 72 h. Luciferase was detected using the Lumat LB 9507 (EG & G Berthold, Bad Wildbad, Germany) and normalized relative to renilla luciferase. The pGL3-basic plasmid served as control in each separate experiment.

**Catalytic glucuronidation assay:** UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10 were transiently transfected into HEK293 cells, cells harvested after 72 hours and used as recombinant protein for UGT catalytic activity assays as previously described in detail (Strassburg, et al., 1999, Strassburg, et al., 2000). Protein amounts were normalized by Western blot using a previously described rabbit anti human UGT1A antibody directed against Exon 2 (Strassburg, et al., 1998). Five mM of SN-38 ((S)-4,11-diethyl-4,9-
dihydro-1H-pyranol [3',4':6,7]indolizino [1,2-b]quinoline-3,14(4H,12H)-dione) (Rhone-Poulenc Rorer, France) was used for each experiment.
Results

Characterization of the UGT1A7 5’ untranslated sequence.

Based upon the genomic DNA sequence deposited in GenBank (accession number AF297093) (Gong, et al., 2001) primers were designed for the amplification of 315 bp upstream of the ATG start codon of the UGT1A7 exon 1 sequence (Figure 1A). The analysis of the obtained sequence suggests that a TATA box for polymerase binding is located between base pairs –59 and –44 from the ATG codon, which is in agreement with the structure of other 5’ untranslated regions at the UGT1A gene locus (Tukey and Strassburg, 2001, Gong, et al., 2001). The analysis of 427 genomic DNA samples from healthy blood donors identified a single nucleotide transversion from T to G at position –57. The homozygous T (-57 T/T) was detected in 160 (37%) individuals, a heterozygous T (-57 T/G) was present in 203 (48%), and the homozygous G (-57 G/G) was identified in 64 (14%) samples (Table 2). Based on these findings –57 T appears to represent the most prevalent (“wildtype”) sequence in our cohort with a gene frequency of 0.61 characterized by a single nucleotide polymorphism with a gene frequency of 0.39. Sequence analysis further indicates that this polymorphism affects the TATA box region of the UGT1A7 gene.

Association of the –57 T>G polymorphism with UGT1A7 exon 1 polymorphisms.

Previous analyses have identified 5 base pair exchanges at positions 11, 129, 131, and 208 in the first exon of UGT1A7 leading to functionally altered UGT1A7 protein variants designated UGT1A7*1 (wildtype), UGT1A7*2, UGT1A7*3 and UGT1A7*4 (Guillemette, et al., 2000, Strassburg, et al., 2002, Zheng, et al., 2001). Studies from different laboratories have found that SNPs at 129 and 131 as well as 11 and 208 appear to be in linkage dyssequilibrium and always occur in combination (Guillemette, et al., 2000, Strassburg, et al., 2002). We therefore studied whether the 5’ untranslated polymorphism at –57bp was associated with the functional SNPs located within exon 1. Taqman allelic discrimination PCR analysis of 427 healthy blood donors was able to precisely discriminate 5’ untranslated and exon SNPs of the UGT1A7 gene (Figure 1 B, C). The data show that –57 G was always present when R208 (T to C transition at codon 208) was detected and never found together with UGT1A7*1 (exon 1) sequence (N129, R131, W208). The T to C exchange at codon 208 of the UGT1A7 first exon is present both in the UGT1A7*3 (K129/K131 and R208) and UGT1A7*4 (N129/R131, W208) genotypes (Table 2). The –57 T/G SNP is therefore in linkage dyssequilibrium with W208R and thus associated with the UGT1A7*3 and
UGT1A7*4 genotypes, which also explains the coincidence of UGT1A7 -57 G with N129K/R131K (Table 2 A). The association of -57 G with the K129/K131 consequently reflects the frequency of the K129/K131, R208 genotype by virtue of its dependency on the presence of R208. As a consequence of this finding –57 G is never present in UGT1A7*1 or UGT1A7*2 unless the patient is compound heterozygous (i.e. UGT1A7*1/*3 etc) (Table 2, A and B). The data was found to be in Hardy-Weinberg equilibrium.

The novel TATA box polymorphism of the UGT1A7 promoter is functional

Both UGT1A7 –57T and UGT1A7 -57G promoter sequence carrying 5’ untranslated sequence fragments were functionally tested in luciferase reporter gene experiments. In six parallel experiments the –57T UGT1A7 putative TATA box construct exhibited 14-fold activation of luciferase expression over control (empty plasmid) in HEK 293 cells (Figure 2 A,B), confirming the presence of a promoter element in the –250bp of the UGT1A7 gene. In contrast, –57 G only showed a 4-fold luciferase expression indicating a 70% reduction of promoter activity attributable to the T to G exchange. Analogous results were also seen in HepG2, Kato III and A549 cells.

Additional mutation analyses revealed that a T to C transition and a T to A transversion showed similarly reduced luciferase activity as -57G (Figure 2B). To characterize the putative TATA box deletion experiments were performed. Deletion of upstream sequence did not change luciferase transcription, but the elimination of the downstream sequence and the TA rich region abolished luciferase activity. This suggests the presence of a TATA box.

The irinotecan metabolite SN-38 is a substrate of the UGT1A1 and UGT1A7 proteins

Recombinant UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, and UGT1A10 were transfected and expressed in HEK293 cells for use in catalytic activity assays. The radiography of the activity assay in Figure 3 demonstrates that UGT1A1, UGT1A6, UGT1A7 and UGT1A10 are identified as relevant isoforms for SN-38 glucuronidation. UGT1A7 showed the highest specific activity with SN-38, which was 5-fold higher than that found with UGT1A1, UGT1A6 and UGT1A10 confirming previous findings (Ciotti, et al., 1999). UGT1A7 is therefore identified as a relevant SN-38 UGT capable of contributing to its toxicity.

Association of the UGT1A7 and the UGT1A1*28 promoter polymorphisms
To elucidate an association UGT1A1*28 as a known risk factor for SN-38 toxicity and then UGT1A7 5’ untranslated variant a cohort of 200 patient DNA samples which were genotyped previously for the presence of Gilbert-Meulengracht’s disease was analyzed (Table 3). In this cohort, sequencing and temperature gradient gel electrophoresis identified 71 patients homozygous for UGT1A1*28, 65 patients heterozygous for UGT1A1*28, and 64 patients with the UGT1A1*1 promoter. Out of the 71 patients who were homozygous for UGT1A1*28 only two displayed UGT1A7 –57 T. One allele of UGT1A7 –57G was therefore present in 97% of subjects. Conversely, individuals with a UGT1A1*1 promoter had a UGT1A7 –57T promoter variant in 73%. These data provide evidence for an association of the Gilbert-Meulengracht promoter UGT1A1*28 with the newly identified functional UGT1A7 promoter polymorphism. Both represent the only known examples of functional promoter polymorphisms at the human UGT1A gene locus. The high activity of UGT1A7 toward SN-38 may indicate a role of this finding for irinotecan efficacy and toxicity in anti-cancer therapy.
Discussion

The precise understanding of human hepatic and extrahepatic drug metabolism is essential for the prediction of disease susceptibility and the efficacy and toxicity of drug therapy (Tukey and Strassburg, 2000, Ehmer, et al., 2004). In this study we identify a functional polymorphism in the upstream region (TA rich element) of the human UGT1A7 gene leading to a 70% reduction of promoter activity. Mutational analyses suggest that –57 T is part of a TATA box element. The identified T to G transversion at position –57 is the second TATA box polymorphism of a human UGT identified to date and provides evidence for yet another mechanism by which human organ-specific glucuronidation is modulated.

In 1995, a TA insertion into the TATA box of the UGT1A1 gene was identified leading to the reduced function A(TA);TAA promoter designated UGT1A1*28. This variant has been found to be associated with unconjugated non-hemolytic hyperbilirubinemia in Gilbert-Meulengracht’s disease (Bosma, et al., 1995, Monaghan, et al., 1996). In subsequent studies UGT1A1*28 was linked to the susceptibility toward breast cancer (Guillemette, et al., 2000). Moreover, it was discovered that one of the principle anti-cancer therapeutics in colorectal cancer, irinotecan, undergoes glucuronidation which involves UGT1A1 but also UGT1A6, UGT1A7 and UGT1A9 (Ciotti, et al., 1999, Gagne, et al., 2002). Therefore, the regulation of UGT1A1 as well as structural gene SNPs of the involved isoforms were hypothesized to influence efficacy and gastrointestinal as well as myelotoxicity of irinotecan therapy. A number of studies have since associated the presence of UGT1A1*28 with irinotecan toxicity (Gagne, et al., 2002, Innocenti, et al., 2004, Iyer, et al., 1999). Additionally the combination of UGT1A1*28 with other coding SNPs such as UGT1A6 were reported (Peters, et al., 2003). However, these analyses have also shown that UGT1A1*28 is not sufficient to explain myelotoxicity and gastrointestinal toxicity in irinotecan-treated patients which also occurs in carriers of the UGT1A1*I gene. A recent analysis of the human UGT1A gene locus has shown that at least 17 SNPs are detectable in the UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9 and UGT1A10 genes (Ehmer, et al., 2004) in addition to 36 variants known for UGT1A1 and the common exon 2-5 domains of the UGT1A genes (Strassburg and Manns, 2000). The combinations of these variants are likely to represent the genetic and biochemical basis of individual drug metabolism by glucuronidation. The present study now introduces a novel variant by identifying an SNP of the UGT1A7 promoter with an allelic frequency of 39% in healthy blood donors. The UGT1A7 gene has been identified as a
risk gene for cancer based on its ability to detoxify benzo(α)pyrene metabolites and heterocyclic amines (Strassburg, et al., 1998) as well as the association of its SNP variants with gastrointestinal and hepatic cancer (Zheng, et al., 2001, Vogel, et al., 2001, Wang, et al., 2004). It is expressed in the proximal gastrointestinal tract in the mouth, esophagus, stomach and in the lung where it establishes contact with airborne, dietary or pharmaceutical xenobiotic compounds including the anti-cancer drug irinotecan and its metabolite SN-38. Recombinant protein experiments (Figure 3) show that SN-38 undergoes significant glucuronidation by UGT1A7 which was found to be 5-fold higher than the catalytic rates detected with UGT1A1, UGT1A6 and UGT1A10. The UGT1A7 promoter variant will reduce this activity by 70%. To complicate the picture, the UGT1A7 variant is in linkage dysequilibrium with the W208R SNP of the UGT1A7 first exon which is present in two UGT1A7 genotypes showing reduced catalytic activity: UGT1A7*3 (N129K/R131K and W208R) and UGT1A7*4 (W208R) (Guillemette, et al., 2000, Strassburg, et al., 2002). Therefore, the presence of the promoter –57 T>G SNP leads to an even further reduction of UGT1A7 activity in its carriers. This finding illustrates that the detection of structural gene SNPs in the UGT1A7 gene and possibly also in other drug metabolizing enzymes is not sufficient to predict the true extent of functional variability which can also be influenced by the presence of SNPs altering the expression of the (“wildtype” or variant) gene product. In view of the known association of irinotecan toxicity with carriers of the UGT1A1*28 genotype we expanded our analysis to patients who exhibit this genotype and found, that among carriers of the UGT1A1*28 allele 98% exhibited at least one allele of the UGT1A7 –57 T>G SNP, and 75% were homozygous for both UGT1A1*28 and UGT1A7 –57 T>G. These data indicate that based on the high specific activity of UGT1A7 for the irinotecan metabolite SN-38 and the association of UGT1A1 and UGT1A7 reduced function promoter polymorphisms the here identified SNP may also play an important role for the understanding and prediction of irinotecan toxicity. Presently, the frequency of -57 T>G in cancer patients alone or in combination with UGT1A1*28 is not known and additional prospective studies will be required to elucidate its potential role as a risk factor for drug associated toxicity. The findings of this study also indicate that search and characterization of SNPs located within the coding exons of drug metabolizing enzymes alone is not sufficient to predict the true picture of altered drug metabolism. Carriers of the most prevalent (“wildtype”) alleles may have regulatory variants which determine their individual metabolic activities. In summary, drug metabolism is altered by (i) individual or combination of individual SNPs located within coding exons, (ii) individual or combination of individual SNPs located in promoter elements,
and (iii) the combination of both. The individual prediction of metabolic predisposition will therefore only be achieved through the analysis of genetic variant patterns (Ehmer, et al., 2004). To this end, relevant candidates such as the promoter SNP identified in this study need to be detected, characterized and assembled into a complex network of determinants of human metabolism.

Beyond the issue of drug therapy the novel promoter SNP offers an explanation for the previously found polymorphic gene regulation detectable for various UGT1A and UGT2B gene products in human stomach (Strassburg, et al., 1998) and human small intestine (Strassburg, et al., 2000). In these tissues individual patterns of UGT mRNA and protein expression were detected. Based on our data, interindividual expression patterns detected at the mRNA steady state level and at the protein level in the presence of normal function DNA variants may be the consequence of SNPs located in the 5’ untranslated regions of the respective genes. Further studies will be required to elucidate the impact of promoter SNPs on the inducibility of drug metabolizing enzymes in different tissues in order to understand the interaction of genetic predisposition and environmental exposure on human metabolism.

In summary, we provide evidence for the second functional promoter polymorphism of a human phase II drug metabolizing enzyme which may influence the clinical finding of irinotecan toxicity. A reduction of promoter activity to 30% of the UGT1A7 –57T gene in addition to the high number and frequency of coding exon SNPs at the human UGT1A gene locus (Ehmer, et al., 2004) conserved during evolution predicts that human glucuronidation is highly individual and requires the establishment of genetic pattern analysis for the prediction of disease predisposition and pharmacogenomically tailored drug therapy. Human glucuronidation can be altered even in the absence of detectable coding region variants of the respective UGT genes studied. The identified UGT1A7 –57T/G TATA box SNP fills a gap in the understanding of UGT regulation and may contribute a useful, facile and inexpensive tool for the prediction and identification of individual disposition.
References


Footnotes:

*Financial support: This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) SFB 621, C3 (to C.P.S.). C.P.S. is supported by a Heisenberg grant from the Deutsche Forschungsgemeinschaft.

Address reprint requests to:
Christian P. Strassburg, M.D.
Associate Professor of Experimental Gastroenterology
Department of Gastroenterology, Hepatology and Endocrinology
Hannover Medical School
Carl-Neuberg-Str. 1
30625 Hannover
Germany

Tel.: x49-511-532 2853
Fax.: x49-511-532 2093
Email: strassburg.christian@mh-hannover.de
Legends for Figures

Figure 1:

**Schematic representation of the UGT1A7 gene upstream sequence.** Shown is a schematic indicating the localization of five exon polymorphisms and the 5’ untranslated polymorphism located at –57 bp upstream of the UGT1A7 gene ATG codon (A). The top left panel shows fluorographs of the different variant sequences. The top right panel shows the results of allelic discrimination PCR analysis (Taqman) capable of discriminating putative TATA box variants. Allelic discrimination of UGT1A7 exon 1 polymorphisms. Shown are typical examples of the allelic discrimination of the SNPs at codon 129/131 (A) and at codon 208 (B) of the UGT1A7 gene by Taqman PCR.

5’-UTR: 5’untranslated region.

Figure 2

**Promoter activity by luciferase reporter gene analysis.** A: Shown is the graphic representation of 6 parallel and independent experiments characterizing the ability of UGT1A7 –57 T and UGT1A7 –57 G promoter sequence to drive luciferase expression in transiently transfected HEK293 cells. Luciferase expression is reduced to 30% in the UGT1A7 –57 G promoter sequence construct. Results are given as means, error bars indicate standard deviations, all results are normalized for renilla activity and are based on experiments with empty vector as controls. Panel B shows mutational analysis of the putative TATA box element representing two individual and parallel experiments. A T to C transition or a T to A transversion leads to reduced luciferase activity comparable to UGT1A7 -57G. Deletion of the upstream sequence retains luciferase activity, but deletion of the putative TATA box or downstream sequence eliminates luciferase transcription. This suggests the presence of an active TATA box element.

Figure 3

**UGT1A7 is the principle SN-38 UGT.** Autoradiography of a catalytic UGT activity assay using recombinant UGT proteins transiently expressed in HEK293 cells and the irinotecan metabolite SN-38 as substrate. Specific UGT activity is strongest for UGT1A7 which is 5-fold higher than the other activities. Protein amounts were normalized by Western blot (not shown). SN38 GLN, glucuronide of the irinotecan metabolite SN-38.
Tables

**TABLE 1**

Primers for Luciferase constructs (A) and Taqman analysis of UGT1A7 single nucleotide polymorphisms (B)

### A

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A7 –57 C F</td>
<td>5’-CTATCTGTACTTCTTTCCACCTACTATATTATAGGAG-3’</td>
</tr>
<tr>
<td>UGT1A7 –57 C R</td>
<td>5’-CTCCTATAAATATAGTAGGTGGAGAAGATAGATAGATAG-3’</td>
</tr>
<tr>
<td>UGT1A7 –57 A F</td>
<td>5’-CTATCTGTACTTCTTTCCACCTACTATATTATAGGAG-3’</td>
</tr>
<tr>
<td>UGT1A7 –57 A R</td>
<td>5’-CTCCTATAAATATAGTAGGTGGAGAAGATAGATAGATAG-3’</td>
</tr>
<tr>
<td>UGT1A7 -57 del upstream F</td>
<td>5’-GAGGGCAGGTTCTATCTTCTTTACTATATTATAGGAGCTTAG-3’</td>
</tr>
<tr>
<td>UGT1A7 -57 del upstream R</td>
<td>5’-CTAAGCTCTATTATATTATAGTAAGAGTGAGTCAAGATGAACCTGCCC C-3’</td>
</tr>
<tr>
<td>UGT1A7 -57 del downstream F</td>
<td>5’-GTTCCATATCCTTCTTTCTTCCACTGAGCTTTGAAACTCCAGCTGCGC-3’</td>
</tr>
<tr>
<td>UGT1A7 -57 del downstream R</td>
<td>5’-GCCAGCAGCTGGGATTCTAAGCTCCAGTGGAAGAAGTGACAGATAAGAAGC-3’</td>
</tr>
<tr>
<td>UGT1A7 -57 del TATA Box F</td>
<td>5’-GGGCAGGTGCTATCTGTACTTCTTCTCTGAGCTTTGGAATGATCCCAGCTAC-3’</td>
</tr>
<tr>
<td>UGT1A7 -57 del TATA Box R</td>
<td>5’-GCACGTGGGATTCTAAGCTCCAGTGGAAGAAGTGACAGATAAGAAGC-3’</td>
</tr>
</tbody>
</table>

### B

| UGT1A7 N129K R131K | Forward Primer | 5’-CACCATTGCGAAGTGCATTT-3’ |
| Reverse Primer | 5’-AGGATGAAAGATCAAA-3’ |
| Probe wildtype | 6-FAM-TAATGACCAAATTT-MGB |
| Probe homozygous | VIC-TTTAAGGACAAAAATTAGT-MGB |

| UGT1A7 W208R | Forward Primer | 5’-CCAGACTTCTCTATTAGGCCTTCAGAC-3’ |
| Reverse Primer | 5’-AGACATTTTTGAAAAATAAGGGGCA-3’ |
| Probe wildtype | 6-FAM-AGGAGAGATGATGACAGA-MGB |
| Probe homozygous | VIC-AGGAGAGATGACAGA-MGB |

| UGT1A7 –57 T>G | Forward Primer | 5’-TTTGGAGGGCAGGTCTCTCTGTA-3’ |
| Reverse Primer | 5’-GCAGCTGGGATTCTAAGCTTACA-3’ |
| Probe wildtype | 6-FAM-TCCTCTCCACTCTATT-MGB |
| Probe homozygous | VIC-TCCTCTCCACTCTATT-MGB |
TABLE 2

Association of 5’ untranslated and exon polymorphisms of the UGT1A7 gene

A

<table>
<thead>
<tr>
<th>UGT1A7 –57 T/G</th>
<th>129N/131R</th>
<th>N129K/R131K (exon 1)</th>
<th>129N131R/129K131K</th>
<th>129K131K</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A7 –57 T</td>
<td>160 (37%)</td>
<td>64 (40%)</td>
<td>75 (47%)</td>
<td>21 (13%)</td>
</tr>
<tr>
<td>UGT1A7 –57 T/G</td>
<td>203 (48%)</td>
<td>0</td>
<td>118 (58%)</td>
<td>85 (42%)</td>
</tr>
<tr>
<td>UGT1A7 –57 G</td>
<td>64 (15%)</td>
<td>0</td>
<td>1 (2%)</td>
<td>63 (98%)</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>UGT1A7 –57 T/G</th>
<th>208W</th>
<th>W208R (exon 1)</th>
<th>208R</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A7 –57 T</td>
<td>160 (37%)</td>
<td>160 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>UGT1A7 –57 T/G</td>
<td>203 (48%)</td>
<td>0</td>
<td>203 (100%)</td>
</tr>
<tr>
<td>UGT1A7 –57 G</td>
<td>64 (15%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Genotyping analyses by Taqman allelic discrimination PCR of the 5’ untranslated SNP, N129K/R131K (A), and W208R (B) variants of the UGT1A7 gene first exon indicate a linkage dysequilibrium of the 5’ untranslated located UGT1A7 –57 G SNP and the exon 1 located 208R SNP. 208R was always present when the UGT1A7 –57 G was detected. Conversely, UGT1A7 –57T was always simultaneously present with 208W. A similar linkage dysequilibrium was not found for N129K/R131K and –57T/G although homozygous carriers of UGT1A7 -57G were also homozygous for the UGT1A7 129N/131K in all but one individual.
TABLE 3

Association of UGT1A1*28 promoter polymorphisms with the novel UGT1A7 promoter polymorphism

<table>
<thead>
<tr>
<th>UGT1A1*28 (A(TA)7TAA)</th>
<th>UGT1A7 –57 G</th>
<th>UGT1A7 –57 T/G</th>
<th>UGT1A7 –57 T</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1*28 (71)</td>
<td>53 (75%)</td>
<td>16 (22%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>UGT1A1*28/*1 (65)</td>
<td>6 (9%)</td>
<td>50 (77%)</td>
<td>9 (14%)</td>
</tr>
<tr>
<td>UGT1A1*1 (64)</td>
<td>5 (8%)</td>
<td>12 (19%)</td>
<td>47 (73%)</td>
</tr>
</tbody>
</table>

Genotyping of 200 patients referred for suspected Gilbert-Meulengracht’s disease. Among these patients 71 were homozygous for the UGT1A7*28 TATA box polymorphism of the UGT1A1 bilirubin transferase gene. Taqman allelic discrimination PCR analysis of all 200 patients demonstrated that in individuals homozygous for the UGT1A1*28 TATA box polymorphism 73% carried the homozygous UGT1A7 –57 G promoter polymorphism, and only 2 (3%) had the “wildtype” UGT1A7 promoter indicating that among homozygous Gilbert patients 98% carry the reduced activity UGT1A7 promoter polymorphism. However, UGT1A7 –57 T>G is also present in individuals who carry a UGT1A1*1 promoter.
Figure 1

A

UGT1A7 -57G/T

FAM Fluorescence

VIC Fluorescence

wildtype
heterozygous
homozygous

0 0.4 0.8 1.2 1.6 1.8

TCTGTACTTCTTCCACCTACTATATTATAGGAAGCTTAGAAATCCACCAGCTGGCTCTGGGCTGAAGTTTCTCTG
TATA Box
5'-UTR
Exon 1

UGT1A7 -57G/T

Codon 11
Silent A/C
N129K R131K
W208R

G/T
Figure 1

UGT 1A7 W208R

FAM Fluorescence

VIC Fluorescence

wildtype

heterozygous

homozygous
Figure 2

A

Fold luciferase activity

UGT1A7-57 T (wildtype)  UGT1A7-57 G (variant SNP)
Figure 2

B

- UGT1A7 -57T
- UGT1A7 -57G
- UGT1A7 -57C
- UGT1A7 -57A
- UGT1A7 -57 del. upstream
- UGT1A7 -57 del. downstream
- UGT1A7 TATA Box Del.

Relative luciferase activity (%)

UGT1A7 –57T: 5'-TCTGTACTTCTTCCACTTACTATATTATAGGAGC -3'
UGT1A7 –57G: 5'-TCTGTACTTCTTCCACGTACTATATTATAGGAGC -3'
UGT1A7 –57C: 5'-TCTGTACTTCTTCCACCTACTATATTATAGGAGC -3'
UGT1A7 –57A: 5'-TCTGTACTTCTTCCACATCTATATTATAGGAGC -3'
UGT1A7 –57 del upstream: 5'-TCTGTACTTC - - - - - - TTACTATATTATAGGAGC -3'
UGT1A7 –57 del downstream: 5'-TCTGTACTTCTTCCACT - - - - - - - - - - - GGAGC -3'
UGT1A7 –57 del TATA 5'-TCTGTACTTC - - - - - - - - - - - - - - - - - GGAGC -3'

This article has not been copyedited and formatted. The final version may differ from this version.