

## Molecular probes for identification of *UDP-glucuronosyltransferase 1* gene polymorphisms for Gilbert's syndrome diagnosis.

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### Abstract

The T-3279G mutation in Phenobarbital Responsive Enhancer Modulein (PBREM), TA-insertion in the TATA box, creating the A(TA)7TAA motif instead of the A(TA)6TAA, and G211A mutation in coding exon 1, particularly in Asians, of human *UGT1A1* (UDP-glucuronosyltransferase) gene are the three common polymorphisms of Gilbert's syndrome. This article employed dot blot hybridization to detect the T-3279G, A(TA)6/7TAA and G211A mutation of *UGT1A1* gene in 15 patients. In the dot blot hybridization assay, PCR products were denatured and spotted on positively charged nylon membranes instead of acrylamide-modified slides. Then three pairs of probes were labeled by digoxigenin and hybridized with the spotted array. After hybridization, the signal was visualized using TMB color development solution and all possible polymorphisms of the T-3279G, A(TA)6/7TAA and G211A in 15 Chinese patients with hyperbilirubinemia were successfully identified. The results of 15 samples by dot-blot hybridization were consistent with the sequencing results. The data showed that dot blot hybridization assay is a quick and low cost method to detect *UGT1A1* gene polymorphisms and helpful to diagnosis of Gilbert's syndrome.

**Keywords:** *UDP-glucuronosyltransferase 1A1*, Molecular probes, Dot blot hybridization, Polymorphisms.

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### Introduction

UDP-glucuronosyltransferases (UGTs), one important family of phase II drug metabolizing enzymes, catalyze the biotransformation of many endobiotics and xenobiotics, and are coded by polymorphic genes [1]. UGT enzymes are mainly expressed in the liver and the intestine and classified into different families, subfamilies and genes. *UGT1A1*, a member of UGT1 subfamily, is a critical enzyme in the elimination pathway of bilirubin. It is well known that inhibition of *UGT1A1*-mediated bilirubin clearance may lead to the elevated level of unconjugated bilirubin in serum, which may lead to hyperbilirubinemia, jaundice, hepatotoxicity and neurotoxicity. Gilbert's syndrome is a mild hereditary unconjugated hyperbilirubinemia due to defect of *IUGT1A1*. Individuals with Gilbert's syndrome exhibit lower glucuronidation rates when medicated with several HIV drugs and irinotecan [2-6].

Numerous polymorphisms of *UGT1A1* have been identified [7,8]. Firstly, a Phenobarbital Responsive Enhancer Module (PBREM) is located approximately 3.5 kbp upstream from the coding region of *UGT1A1* gene and the T-3279G mutation in PBREM is a common polymorphism causing Gilbert syndrome [9]. Secondly, the *UGT1A1*\*28 polymorphism is characterized by the TA-insertion in the

TATA box, creating the A(TA)7TAA motif instead of the A(TA)6TAA. This thymine- and adenine-rich regulatory region is the major recognition point for the binding of transcription factors and RNA polymerase in many genes. Clinical studies have demonstrated a significant correlation between the *UGT1A1*\*28 polymorphism and the pharmacokinetics of drugs [10-12]. Finally, a significant association was observed between prolonged jaundice in male infants and *UGT1A1* G71R polymorphism (G211A mutation in coding exon 1 of *UGT1A1* gene) [13]. The coding region polymorphism is found only in Asians and is associated with decreased enzyme function, whereas the promoter polymorphism is associated with decreased gene expression. Individuals carriers of the *UGT1A1*\*6 (G211A mutation) and \*28 variants are apparently much more likely to develop jaundice after medication with methotrexate, indinavir, sorafenib, atazanavir or other drugs inhibiting *UGT1A1*. This is presumably due to the lower functional reserve available to compensate *UGT1A1* inhibition [6].

To our knowledge, no study has analyzed the relationship between *UGT1A1* polymorphisms and dot blot hybridization assay with molecular probes. In the dot blot hybridization assay, the obtained PCR products were successively purified denatured, and then digoxigenin-labeled probes were allowed

to hybridize to DNA array. The hybridization results could be determined by visualizing the DNA array using bioluminescence method.

Previously, we applied three dimensional acrylamide gel method to detect *UGT1A1* gene polymorphism [14]. However, preparation of the acrylamide-modified slides is time-consuming. Fluorescent labeled probes, acrylamide-modified primers and the use of Luxscan-10K/A (CapitalBio Company, China) are expensive. Other method, such as direct sequencing method, TaqMan MGB SNP genotyping assay, DNA melting curve analysis, Restriction Fragment Length Polymorphism (RFLP) method have been used to detect mutations of *UGT1A1* gene for Gilbert's Syndrome diagnosis [15-17]. In this study, we applied dot blot hybridization assay with molecular probes for the first time to detect the T-3279G, A(TA)<sub>6</sub>/TAA and G211A mutation of *UGT1A1* gene.

## Materials and Methods

### Participants and DNA isolation

15 Chinese patients with hyperbilirubinemia were recruited at the Second Affiliated Hospital of Southeast University, Nanjing, P. R. China. All patients had total bilirubin levels (TBIL) of  $\geq 220.6$   $\mu\text{mol/L}$ , and no signs or symptoms indicative of other hepatobiliary diseases. Serum samples were used for the evaluation of total bilirubin level, and all biochemical tests were carried out in the same laboratory with standardized laboratory methods. Peripheral blood samples were collected from all participants in the morning after overnight fasting. Total DNA was extracted using the QIAamp DNA midi blood kit (QIAGEN, Germany) according to the standard protocol. All participants provided a written informed consent before enrolment and all research procedures were approved by the Ethics Committee of the Second Affiliated Hospital of Southeast University.

### PCR amplification

A pair of primers F1 (5'-CACCTCCTCCTTATTCTCTT-3') and R1 (5'-CTCATTCCTCCTCTCTAGCC-3'), whose design was based on published DNA sequence, was used in the PCR to obtain the PBREM region of *UGT1A1* gene. The procedure was as follows: 94°C for 3 min, 32 cycles of 94°C for 30 s, 54.2°C for 45 s, and 72°C for 45 s, and then 72°C for 10 min. The region containing TATA-box and 211 site of *UGT1A1* gene was generated by PCR using Ex Taq (Takara, Japan) with two primers F2 (5'-CCCTGCTACCTTTGTGGACT-3') and R2 (5'-CATTATGCCCGAGACTAACAAA -3'). The reaction conditions were 94°C for 3 min followed by 32 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s, and then a final elongation step at 72°C for 10 min. After agarose gel electrophoresis analysis, PCR products were processed by ethanol precipitation overnight at -20°C. Then, PCR products were harvested by centrifugation at 14000 g for 20 min and diluted in water.

### PCR products immobilization

PCR products were denatured by mixing with the same volume of denaturation solution (0.5 N NaOH, 1.5 M NaCl) at room temperature for 30 min. The denatured DNAs were then spotted on 25 mm positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany), followed by baking at 100°C for 30 min.

### Hybridization with the corresponding probes

The spotted array was pre-hybridized with 1  $\times$  DIG Easy Hyb (Roche Diagnostics) in a hybridization oven (Robbins Scientific Corp., USA) for 4 h at 60°C. Samples of 10 ml (50 ng) of DIG-labeled candidate probe (Table 1) was denatured for 10 min at 98°C and mixed with 1  $\times$  DIG Easy Hyb (1.5 ml) warmed to 60°C. The mixture was incubated with nylon membranes bearing DNA arrays overnight at 37°C in the hybridization overnight.

**Table 1.** The probe sequences used in this study.

Probe	Probe sequences
-3279T	5'-Digoxigenin-TTCAGTTTGAACA-3'
-3279G	5'-Digoxigenin-TTCAGTGTGAACA-3'
A(TA) <sub>6</sub> TAA	5'-Digoxigenin-GCCATATATATATATAAG-3'
A(TA) <sub>7</sub> TAA	5'-Digoxigenin-GCCATATATATATATATAAG-3'
211G	5'-Digoxigenin-AGAG ACGGAGCAT-3'
211A	5'-Digoxigenin-AGAGACAGAGCAT-3'

Bold under led characters indicate the test loci.

### Image scanning

After hybridization, the nylon membranes were washed twice with 50 ml of 23 SSC/0.1% SDS at room temperature, each for 10 min, and twice with 50 ml of 0.53 SSC/0.1% SDS at 62°C, each for 10 min, respectively. Then the nylon membranes were blocked with 10% BSA for 1 h. The membranes were incubated with a 1:1000 dilution of Horseradish Peroxidase-conjugated IgG Fraction Monoclonal Mouse Anti-Digoxin Antibody (Jackson, USA) for 30 min and then washed three times with TBST buffer (20 mM Tris HCl pH 8.0, 150 mM NaCl, and 0.05% (v/v) Tween 20). Finally, the signal was visualized using TMB color development solution (Phygene, China).

## Results

The general clinical data are shown in Table 2. 15 patients included 10 males and 5 females with a median age of 55 y (range, 38-68). All patients had TBIL of  $\geq 220.6$   $\mu\text{mol/L}$ . In principle, two rounds of dot-blot hybridization were carried out simultaneously.

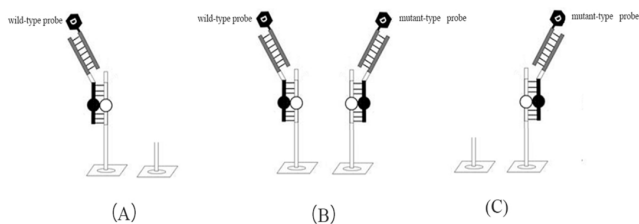
**Table 2.** General clinical data in the 15 patients.

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Patient	Gender	Age	TBIL (μmol/L)	DBIL (μmol/L)	IBIL (μmol/L)
1	Male	56	300.4	36	264.4
2	Male	60	287.1	15.6	271.5
3	Male	48	252	9.5	242.5
4	Female	45	312.5	33.2	279.3
5	Female	68	227.1	6	221.1
6	Male	43	280	12.6	267.4
7	Male	62	336.8	38	298.8
8	Female	53	325	35	290
9	Male	66	246.9	5.9	241
10	Male	55	288.1	22.3	265.8
11	Female	58	252	7.9	244.1
12	Female	47	308.4	22.3	286.1
S1	Male	61	273.3	13	260.3
S2	Male	56	268.9	8.7	260.2
S3	Male	38	319.6	30.9	288.7

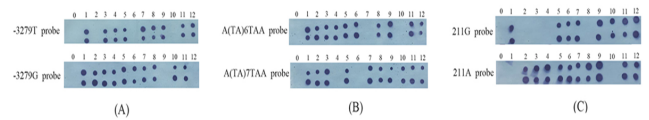
TBIL: Serum Total Bilirubin; DBIL: Conjugated Bilirubin; IBIL: Unconjugated Bilirubin.

The homozygous wild type hybridized with only digoxigenin-labeled wild-type probes, showing wild-type signals. While the homozygous mutant hybridized with only digoxigenin-labeled mutant-type probes, showing mutant signals. Both the wild-type signals and the mutant signals were shown for the heterozygote. So, mutant alleles were distinguished from wild-type alleles. A schematic outline of genotyping approach using dot-blot hybridization is shown in Figure 1.

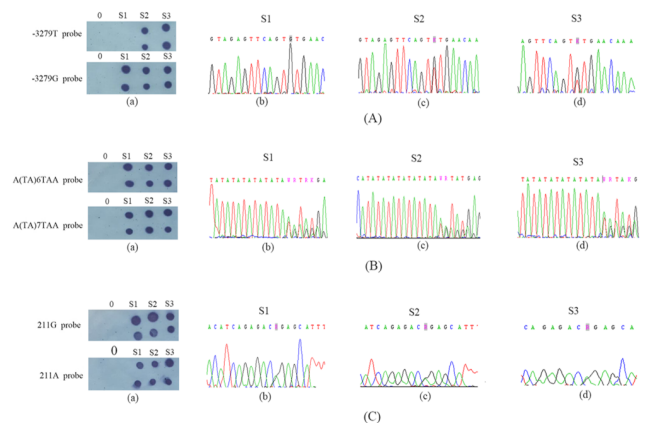


**Figure 1.** A schematic outline of genotyping approach using dot-blot hybridization. (A) Homozygous wild type; (B) Heterozygote; (C) Homozygous mutant.

12 samples were screened by dot-blot hybridization. All possible genotypes of 12 samples were successfully identified and shown in Figure 2. Images from 12 samples assayed for the T-3279G, A(TA)6/7TAA, G211A of the *UGT1A1* were shown in Figures 2 (A)-2(C), respectively. In addition, 3 patients were also screened by dot-blot hybridization and sequencing results of the PCR products were shown in Figure 3. Hybridization results and sequencing results of T-3279G, A(TA)6/7TAA, G211A in *UGT1A1* gene of 3 additional patients were shown in Figures 3(A)-3(C), respectively. Variations of *UGT1A1* genotypes in 15 patients are shown in Table 3.



**Figure 2.** (A) Images from 12 samples assayed for the T-3279G of the *UGT1A1* gene. 0, blank control, genotypes of 1-15 are shown in Table 3; (B) Images from 12 samples assayed for the A(TA)6/7TAA of the *UGT1A1* gene. 0, blank control, genotypes of 1-15 are shown in Table 3; (C). Images from 12 samples assayed for the G211A of the *UGT1A1* gene. 0, blank control, genotypes of 1-15 are shown in Table 3. Each sample was spotted two times in a row.



**Figure 3.** (A) Hybridization results and sequencing results of T-3279G in *UGT1A1* gene of 3 additional patients. (a) Hybridization results of T-3279G in *UGT1A1* gene. 0, blank control, genotypes of S1-S3 are shown in Table 3; (B) Hybridization results and sequencing results of A(TA)6/7TAA in *UGT1A1* gene of 3 additional patients. (a) Hybridization results of A(TA)6/7TAA in *UGT1A1* gene. 0, blank control, genotypes of S1-S3 are shown in Table 3. (b) Sequencing results of A(TA)6/7TAA in *UGT1A1* gene of S1 patients. (c) Sequencing results of A(TA)6/7TAA in *UGT1A1* gene of S2 patients. (d) Sequencing results of A(TA)6/7TAA in *UGT1A1* gene of S3 patients; (C) Hybridization results and sequencing results of G211A in *UGT1A1* gene of 3 additional patients. (a) Hybridization results of G211A in *UGT1A1* gene. 0, blank control, genotypes of S1-S3 are shown in Table 3. (b) Sequencing results of G211A in *UGT1A1* gene of S1 patients. (c) Sequencing results of G211A in *UGT1A1* gene of S2 patients. (d) Sequencing results of G211A in *UGT1A1* gene of S3 patients. Each sample was spotted two times in a row.

**Table 3.** Variations of *UGT1A1* genotypes in 15 patients.

Patient	T-3279G	A(TA)6/7TA	G211A
1	heterozygote T-3279G	heterozygote A(TA)6/7TAA	homozygote G211G
2	homozygote G-3279G	heterozygote A(TA)6/7TAA	homozygote A211A
3	heterozygote T-3279G	heterozygote A(TA)6/7TAA	homozygote A211A
4	heterozygote T-3279G	homozygote A(TA)6TAA	homozygote A211A
5	heterozygote T-3279G	heterozygote A(TA)6/7TAA	heterozygote G211A

6	homozygote G-3279G	homozygote A(TA)6TAA	heterozygote G211A
7	heterozygote T-3279G	homozygote A(TA)7TAA	heterozygote G211A
8	heterozygote T-3279G	heterozygote A(TA)6/7TAA	homozygote A211A
9	homozygote T-3279T	heterozygote A(TA)6/7TAA	heterozygote G211A
10	homozygote G-3279G	homozygote A(TA)7TAA	homozygote G211G
11	heterozygote T-3279G	heterozygote A(TA)6/7TAA	heterozygote G211A
12	homozygote T-3279T	heterozygote A(TA)6/7TAA	heterozygote G211A
S1	homozygote G-3279G	heterozygote A(TA)6/7TAA	heterozygote G211A
S2	heterozygote T-3279G	heterozygote A(TA)6/7TAA	heterozygote G211A
S3	heterozygote T-3279G	heterozygote A(TA)6/7TAA	heterozygote G211A

## Discussion

*UGT1A1* is the only physiologically relevant UGT isoform involved in the metabolic clearance of endobiotics bilirubin. Potent inhibition of *UGT1A1* may result in partial or complete loss of *UGT1A1* activity, and then increase the exposure of bilirubin and other endogenous (e.g., steroid hormones and bile acids) and xenobiotics (e.g., drugs, chemical carcinogens, and environmental pollutants), which may lead to a series of undesirable effects including jaundice, liver dysfunction and carcinogenesis. Several studies show that tolcapone, entacapone, licochalcone A, fructus psoraleae, amentoflavone displayed strong inhibitory effects on human *UGT1A1* [1,18-20]. These findings provide a deep understanding of potential drug interactions between above mentioned drugs and *UGT1A1* substrates via *UGT1A1* inhibition, and will be helpful for guiding reasonable applications of above mentioned drugs and its related medical preparations in the future.

In our paper, products were denatured and spotted on positively charged nylon membranes instead of acrylamide-modified slides. After positively charged nylon membranes were baked at 100°C, some of the pyrimidine bases in the nucleic acid can be combined with the positive charges on the membranes. In the process of PCR products immobilization, no toxic substances were introduced, such as acrylamide monomer and TEMED. According to the three-dimensional polyacrylamide gel microarray technique, preparation of the acrylamide-modified slides is time-consuming [21]. Fluorescent labeled probes, acrylamide-modified primers and the use of Luxscan-10K/A (CapitalBio Company, China) are expensive. Based on the three-dimensional polyacrylamide gel microarray technique, dot blot hybridization, which is a safe, no toxicity and lower cost method, was used in our study. Although, dot blot hybridization analysis requires some labor for the detection of the allele-specific signals, this method is

suitable for a large number of samples because a large number of samples can be blotted on one low-priced membrane and the costliest process in dot blot hybridization analysis is DIG-labeled probes.

In summary, we have successfully detected the *UGT1A1* gene mutations in 15 Chinese patients with hyperbilirubinemia through dot blot hybridization method. This method will hold significant promise for applications in detection of *UGT1A1* gene polymorphisms.

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