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A Polymorphic Mutation, c.-3279T>G, in the *UGT1A1* Promoter Is a Risk Factor for Neonatal Jaundice in the Malay Population

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ABSTRACT: The uridine diphosphoglucuronate-glucuronosyltransferase 1A1 (*UGT1A1*) gene encodes the enzyme responsible for bilirubin glucuronidation. To evaluate the contribution of *UGT1A1* promoter mutations to neonatal jaundice, we determined the genotypes of c.-3279T>G, c.-3156G>A, and A(TA)7TAA in Malay infants with neonatal jaundice (patients) and in infants without neonatal jaundice (controls). In our population study, only c.-3279T>G was associated with neonatal jaundice. The genotype distributions between both groups were significantly different ($p = 0.003$): the frequency of homozygosity for c.-3279G was much higher in patients than those in controls. Allele frequency of c.-3279G was significantly higher in patients than those in controls ($p = 0.006$). We then investigated changes in transcriptional activity because of c.-3279T>G. Luciferase reporter assay in HepG2 cells demonstrated that transcriptional activity of the c.-3279G allele was significantly lower than that of the c.-3279T allele in both the absence and presence of bilirubin. Luciferase reporter assay in COS-7 cells elucidated that c.-3279T>G modified the synergistic effects of the nuclear factors associated with transcriptional machinery. In conclusion, the c.-3279T>G mutation in the *UGT1A1* promoter is a genetic risk factor for neonatal jaundice. **(*Pediatr Res* 67: 401–406, 2010)**

The uridine diphospho (UDP)-glucuronate-glucuronosyltransferase 1A1 (*UGT1A1*) gene encodes bilirubin UDP-glucuronosyltransferase (*UGT1A1*), which is the enzyme responsible for bilirubin glucuronidation. A decrease in *UGT1A1* activity leads to unconjugated hyperbilirubinemia. Inherited *UGT1A1* deficiencies because of *UGT1A1* mutations are classified into three forms based on clinical severity: Crigler-Najjar syndrome type 1 (CN-1, a severe form), Crigler-Najjar syndrome type 2 (CN-2, an intermediate form), and Gilbert's syndrome (GS, a mild form) (1,2). Such inherited *UGT1A1* deficiencies may cause neonatal jaundice.

A mutant TATA box with an additional TA insertion, A(TA)7TAA, was first found in patients with CN-1, CN-2, and GS (3–5). GS with homozygosity for A(TA)7TAA accelerates or prolongs neonatal jaundice (6). This mutation is frequently observed in whites and Africans (7). Another GS-causing gene mutation, c.211G>A, is frequent in the East Asian population (8). The c.211G>A mutation causes an amino acid change, glycine to arginine at codon 71.

Recently, polymorphic mutations, c.-3279T>G and c.-3156G>A, were identified in the *UGT1A1* promoter region (9,10). The c.-3279T>G mutation is located in the phenobarbital responsive enhancer module (gtPBREM), which is activated by a nuclear receptor, constitutive androstane receptor (CAR) (11). Sugatani *et al.* (9) showed using luciferase reporter assay that mutant gtPBREM with c.-3279T>G decreases the transcriptional activity of the *UGT1A1* promoter by 60%. The c.-3156G>A mutation is located between the gtPBREM and TATA box. A haplotype analysis showed that there is a high extent of linkage disequilibrium between c.-3156G>A and A(TA)7TAA (10,12). However, it is unknown whether c.-3156G>A reduces the transcriptional activity of the *UGT1A1* promoter.

Although several studies of the A(TA)7TAA and c.211G>A mutations in the Southeast Asian population have been reported (13,14), the c.-3279T>G and c.-3156G>A mutations have never been examined in this population. Therefore, we determined the genotypes of the mutations, c.-3279T>G, c.-3156G>A, and A(TA)7TAA, in Malay infants with or without neonatal jaundice to evaluate the contribution of the mutations of the *UGT1A1* promoter to the development of neonatal jaundice. In addition, we conducted a luciferase reporter assay with gtPBREM with or without

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Abbreviations: AhR, human aryl hydrocarbon receptor; **Arnt,** human aryl hydrocarbon receptor nuclear translocator; **CAR,** human constitutive androstane receptor; **GS,** Gilbert's syndrome; **gtPBREM,** Phenobarbital responsive enhancer module in *UGT1A1*; **RXR,** human retinoid X receptor α; **UGT1A1,** uridine diphosphoglucuronate-glucuronosyltransferase 1A1

c.-3279T>G, to examine the effect of this mutation on transcriptional activity of the *UGT1A1* gene.

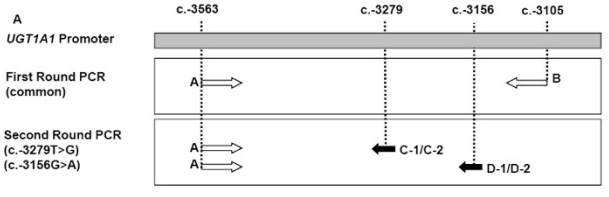
SUBJECTS AND METHODS

Subjects. In Kelantan, Malaysia, the Malays are the dominant population. Based on data of the Neonatal Ward at University Sains Malaysia (USM) Hospital in Kelantan, the overall rate of neonatal jaundice is ~10% in Malay infants. In this study, 136 Malay infants in USM hospital with a gestational age of >37 wk and birth weights of >2500 g were enrolled. Written informed consent was obtained from their parents. The subjects were divided into two groups: the jaundice group (n = 66) and control group (n = 70). A serum bilirubin (total) concentration of >15 mg/dL (250 μM) within the first week after birth was defined as neonatal jaundice.

Infants fulfilling any of the following criteria were excluded from the study as follows: prematurity, presence of gross congenital malformations, Coombs-positive hemolytic anemia, birth trauma causing extravasation of blood (such as cephalohematoma or subaponeurotic hemorrhage), polycythemia, maternal diabetes, positive serology for viral hepatitis, or evidence of sepsis or asphyxia. Three infants with glucose-6-phosphate dehydrogenase (G6PD) deficiency and three infants with Southeast Asian ovalocytosis (SAO) were included in the jaundice group, but there were no infants with G6PD deficiency or SAO in controls. However, information on ABO and Rh blood types was not available in all subjects. This study was approved by the ethical committees of USM and Kobe University.

Detection of c.-3279T>G, c.-3156G>A, and A(TA)7TAA mutations. Genomic DNA was extracted from whole blood of the infants. To detect the c.-3279T>G and c.-3156G>A mutations, we conducted a combination method of semi-nested PCR and competitive oligonucleotide priming (COP)-PCR. Semi-nested PCR can avoid unexpected amplification, and the COP-PCR method with a short primer can detect a single nucleotide change (15). The primer sequences are listed in Figure 1. The first round of PCR amplified the target region including nucleotide positions c.-3279 and c.-3156, and the second round of PCR specifically amplified fragments with c.-3279T, c.-3279G, c.-3156G, and c.-3156A. In the second round, COP-PCR was conducted with a common forward primer used in the first round PCR and a reverse primer, which specifically bound to one of the variant nucleotides. The annealing temperatures of COP-PCR were as follows: 42°C for c.-3279T, 44°C for c.-3279G, 30°C for c.-3156G, and 32°C for c.-3156A. To confirm the COP-PCR, we sequenced all of the amplified fragments of the first round PCR by a dye-terminator automated sequencing method. To detect the A(TA)7TAA mutation, we determined the PCR fragment length as described previously (13).

Haplotype analysis. The *UGT1A1* haplotypes were statistically inferred using an algorithm based on Bayesian inference, PHASE version 2.1.1 (http://www.stat.washington.edu/stephens), with a fair degree of precision.



B

PCR	Type	Name	Sequence
First Round PCR	Forward	Primer A (common)	5'-CAC TCA ATT CCA AGG GGA AA-3'
	Reverse	Primer B (outer)	5'-AGT CCT CTG CCT TGC TCT CA-3'
Second Round PCR for c.-3279T>G	Forward	Primer A (common)	5'-CAC TCA ATT CCA AGG GGA AA-3'
	Reverse	Primer C-1 (inner for c.-3279T)	5'-GTT CAA ACT G-3'
		Primer C-2 (inner for c.-3279G)	5'-GTT CAC ACT G-3'
Second Round PCR for c.-3156G>A	Forward	Primer A (common)	5'-CAC TCA ATT CCA AGG GGA AA-3'
	Reverse	Primer D-1 (inner for c.-3156G)	5'-CTG TCC AAG CTC-3'
		Primer D-2 (inner for c.-3156A)	5'-CTG TCT AAG CTC-3'

Figure 1. Semi-nested PCR was used to detect c.-3279T>G and c.-3156G>A mutations in *UGT1A1*. *A*, Primer locations. The first PCR amplified the region of interest, including nucleotide positions c.-3279 and c.-3156, and the second PCR specifically amplified fragments with c.-3279T, c.-3279G, c.-3156G, and c.-3156A. *B*, Primer sequences. The length of nucleotide-specific reverse primers used in the second PCR was only 11–12 mer. Such short primers enabled nucleotide-specific amplification.

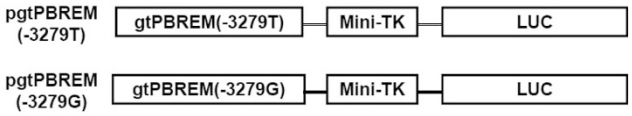


Figure 2. Construction map of pgtPBREM (gtPBREM-pGL2-Basic-miniTK plasmid). The 290-bp amplified *UGT1A1* PBREM fragments (gtPBREM) with c.-3279T and c.-3279G were inserted into a pGL2-Basic vector with a minimum HSV-tyrosine kinase promoter sequence (Mini-TK). LUC, luciferase gene (firefly).

The haplotypes were inferred by running PHASE for a total of 10 times, and the relative SD of their frequencies was 5% or less of the mean value (16,17).

Preparation of expression vectors. The gtPBREM-TK-firefly luciferase expression plasmid was prepared according to the method of Sugatani *et al.* (9,11). The PCR-amplified *UGT1A1* PBREM fragments (gtPBREM) with c.-3279T and c.-3279G (290 bp) were inserted into a luciferase reporter plasmid, pGL2-Basic-miniTK (pGL2-Basic vector with a minimum HSV-tyrosine kinase promoter sequence). These gtPBREM-TK-firefly luciferase expression plasmids were designated as "pgtPBREM(-3279T)" and "pgtPBREM(-3279G)." The construct maps of pgtPBREMs are presented in Figure 2.

The human CAR expression plasmid, pCAR, was prepared using Gateway Technology (Invitrogen/Life Technologies Corporation, CA). The CAR cDNA sequence was obtained by PCR amplification using a human liver cDNA library (BioChain Institute, Inc., CA). The human retinoid X receptor α (RXR) expression plasmid, pRXR, was kindly provided by Dr. Ron M. Evans (The Salk Institute for Biological Studies, CA). The human aryl hydrocarbon receptor (AhR) and human aryl hydrocarbon receptor nuclear translocator (Arnt) expression plasmids, pAhR and pArnt, were kindly provided by Dr. Susan Moran and Dr. Christopher Bradfield (McArdle Laboratory for Cancer Research, University of Wisconsin-Madison Medical School, WI). All expression vectors were sequenced by dye-terminator automated sequencing as described above.

Transcriptional activity assay. In this study, the response of mutant pgtPBREM to bilirubin was determined with HepG2 cells, and overexpression effects of "CAR and RXR," "AhR and Arnt," and "CAR, RXR, AhR, and Arnt" were examined in COS-7 cells.

The gtPBREM fragment is a composite regulatory element containing the multiple binding sites, gtNR1 (for nuclear receptors overexpressed in the cells in this study, CAR and RXR), xenobiotic responsive element (XRE; for AhR and Arnt), and NR3 (for CAR) (12,18,19).

In the first assay, the HepG2 cells [2×10^5 cells in Minimum Essential Medium (MEM)] were cotransfected with pgtPBREM plasmid (0.75 μg) and phRL (sea pansy) plasmid (0.1 μg) (Promega Corporation, WI) using Lipofectamine 2000 (Invitrogen/Life Technologies Corporation). Twenty-four hours after transfection, bilirubin (15 μM) was added to the MEM growing medium. The cells were then harvested after an additional 24 h of culture in the presence of bilirubin.

In the second assay, the COS-7 cells (2×10^5 cells in MEM) were cotransfected with pgtPBREM(-3279T) or pgtPBREM(-3279G) plasmids (0.75 μg), pAhR + pArnt plasmids (0.25 μg each) and/or pCAR + pRXR plasmids (0.25 μg each), and phRL plasmid (0.01 μg) (Promega, Madison, WI) using Lipofectamine 2000. The cells were harvested 48 h after transfection. Transcriptional activity was measured simultaneously using the dual-luciferase reporter assay system, in which sea pansy-luciferase activity of the phRL plasmid was used as an internal control for the transfection efficiency of pgtPBREM plasmids.

Statistical analysis. The allele frequencies were examined for Hardy-Weinberg equilibrium by χ^2 and Fisher's exact tests. The differences in genotype and allele frequencies between the neonatal jaundice and control groups were also examined using the χ^2 test and Fisher's exact test. Experiments were repeated in triplicate in the transcriptional activity assay. The transcriptional activities were compared by t tests. p values of <0.05 were considered significant.

RESULTS

PCR-based techniques for mutation detection. The second round PCR (COP-PCR) specifically amplified the fragments with c.-3279T or c.-3279G (221 bp) and the fragments with

c.-3156G or c.-3156A (334 bp). The gel electrophoresis clearly separated each genotype (Fig. 3). The sequencing data were consistent with the PCR results.

To detect the TATA box mutation, A(TA)7TAA, we determined the length of the PCR fragment of the *UGT1A1* promoter including the TATA box according to the GeneScan method described elsewhere (13). GeneScan analysis showed two types of fragments: a 90-bp fragment with the normal TATA box A(TA)6TAA and a 92-bp fragment with the mutated TATA box A(TA)7TAA (Fig. 3).

c.-3279T>G, c.-3156G>A, and A(TA)7TAA mutations. In Malay infants, c.-3156A was always found with A(TA)7TAA, suggesting a close linkage between them. However, there was not a close linkage between c.-3279G and c.-3156A, or between c.-3279G and A(TA)7TAA. These findings were confirmed by haplotype analysis using PHASE software (see below).

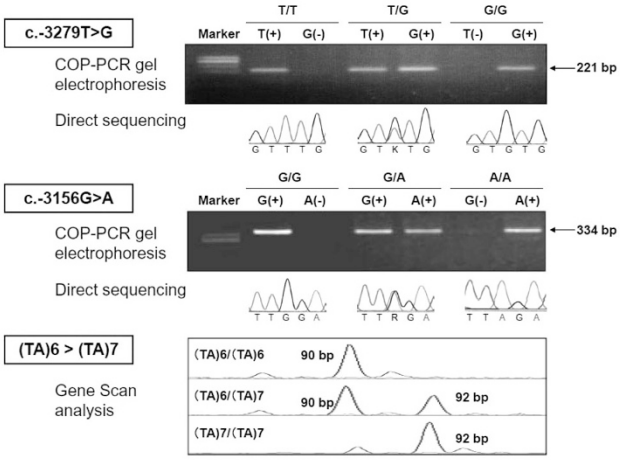


Figure 3. Detection of c.-3279T>G, c.-3156G>A, and A(TA)7TAA. Two percent agarose gel electrophoresis of COP-PCR products was used to show each genotype for c.-3279T>G and c.-3156G>A. Phi-X 174 DNA-HaeIII digest was used as a marker. To confirm the COP-PCR data, we performed direct sequencing of the first round PCR products. "K" denotes T and G and "R" denotes G and A in the sequencing data. GeneScan analysis was used to show each genotype for A(TA)6TAA>A(TA)7TAA. (TA)6, A(TA)6TAA; (TA)7, A(TA)7TAA.

Genotype distributions based on the nucleotide at c.-3279 are shown in Table 1. The genotype distributions of jaundice and control groups did not shift from Hardy-Weinberg equilibrium. The genotype distributions between both groups were significantly different ($p = 0.003$): the frequency of homozygosity for c.-3279G was much higher in patients than those in controls. The allele frequency of c.-3279G was also significantly higher in jaundice infants than that in controls ($p = 0.006$).

Genotype distributions based on the nucleotide at c.-3156 and the TA repeat number are also shown in Table 1. The genotype distributions based on both polymorphisms in the jaundice and control groups did not shift from Hardy-Weinberg equilibrium. There were no significant differences in genotype distribution or allele frequency between the groups.

Clinical features among genotypes based on the nucleotide at c.-3279. There was no significant difference in the onset time of hyperbilirubinemia among the newborn infants with the different genotypes. With regard to the duration of hyperbilirubinemia, we were not able to come to any conclusion because phototherapy was given to all the newborn infants with hyperbilirubinemia.

There was no significant difference in mean peak bilirubin levels in jaundice infants among the different genotypes based on the nucleotide at c.-3279: T/T, 23.4 ± 4.3 mg/dL (mean ± SD); T/G, 23.3 ± 5.1 mg/dL; and G/G, 23.0 ± 5.0 mg/dL. There was also no significant difference in bilirubin levels in control infants among the different genotypes based on the nucleotide at c.-3279: T/T, 9.8 ± 2.3 mg/dL; T/G, 10.6 ± 2.0 mg/dL; and G/G, 10.9 ± 1.1 mg/dL.

Haplotype analysis in the UGT1A1 promoter region using PHASE software. Table 2 shows the estimated frequencies of the *UGT1A1* promoter haplotypes in jaundice and control groups. Three major haplotypes were statistically inferred with a frequency of >10% in both groups. With these major haplotypes, the frequency reached a total of ~95% in each group. However, the haplotype distributions were significantly different from each other ($p = 0.016$). The frequency of

Table 1. *Genotype and allele frequencies of UGT1A1 promoter mutations*

	Genotype frequency				Allele frequency		
	T/T	T/G	G/G	Total	T	G	Total
c.-3279T>G							
Neonatal jaundice	19 (29%)	29 (44%)	18 (27%)	66 (100%)	67 (51%)	65 (49%)	132 (100%)
Control	28 (40%)	38 (54%)	4 (6%)	70 (100%)	94 (67%)	46 (33%)	140 (100%)
				$p = 0.003$			$p = 0.006$
	G/G	G/A	A/A	Total	G	A	Total
c.-3156G>A							
Neonatal jaundice	51 (77%)	14 (21%)	1 (2%)	66 (100%)	116 (88%)	16 (12%)	132 (100%)
Control	51 (73%)	16 (23%)	3 (4%)	70 (100%)	118 (84%)	22 (16%)	140 (100%)
				$p = 0.73$			$p = 0.39$
	(TA)6	(TA)6/(TA)7	(TA)7	Total	(TA)6	(TA)7	Total
TATA box							
Neonatal jaundice	51 (77%)	14 (21%)	1 (2%)	66 (100%)	116 (88%)	16 (12%)	132 (100%)
Control	53 (76%)	16 (23%)	1 (1%)	70 (100%)	122 (87%)	18 (13%)	140 (100%)
				$p = 0.97$			$p = 0.85$

Table 2. *Estimated frequencies of UGT1A1 promoter mutations in Malay infants*

Position			Estimated frequency (%)*		
c.-3279	c.-3156	TATA box	Jaundice	Control	p†
T	G	(TA) 6	50.0	66.0	0.016
G	G	(TA) 6	37.0	19.0	
G	A	(TA) 7	11.0	13.0	
G	A	(TA) 6	0.8	1.0	
T	G	(TA) 7	0.8	—‡	
T	A	(TA) 6	—‡	2.0	
G	G	(TA) 7	—‡	—‡	
T	A	(TA) 7	0.1	—‡	

* *UGT1A1* haplotypes were statistically inferred using an algorithm based on Bayesian inference, PHASE version 2.1.1 (http://www.stat.Washington.edu/stephens/).

† Haplotype frequency comparisons between neonatal jaundice and control groups.

‡ –Not inferred.

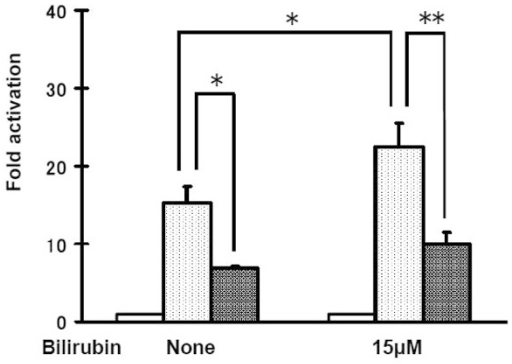


Figure 4. Luciferase reporter assay in HepG2 cells. Transcriptional activity of normal pgtPBREM(-3279T) and mutant pgtPBREM(-3279G) in HepG2 cells was determined in the presence of bilirubin and its possible oxidized product in the medium. The luciferase activity of pGL2-Basic-miniTK was denoted as 1-fold activation. □, pGL2-Basic-miniTK (Mock); ▨, pgtPBREM (-3279T); and ▩, pgtPBREM(-3279G). *$p < 0.05$ and **$p < 0.01$.

haplotype [-3279G/-3156G/(TA)6] in the jaundice group was twice that in controls, whereas the frequency of haplotype [-3279G/-3156A/(TA)7] was similar between the groups.

Transcriptional activity assay of normal and mutant pgtPBREMs. First, we determined the transcriptional activity of pgtPBREM(-3279T) and pgtPBREM(-3279G) in HepG2 cells in both the absence and presence of bilirubin (and its possible oxidized product) in the medium. There was a significant difference between the transcriptional activities of both pgtPBREM plasmids ($p = 0.003$ in the absence of bilirubin and $p = 0.003$ in the presence of bilirubin) (Fig. 4).

The pgtPBREM(-3279T) promoter was significantly activated by the addition of bilirubin in the medium (15 μM) ($p = 0.028$), whereas the pgtPBREM(-3279G) promoter was not significantly activated (Fig. 4). In the absence of bilirubin, the transcriptional activity of the pgtPBREM(-3279G) promoter was ~50% of that of the pgtPBREM(-3279T) promoter. In the presence of bilirubin, however, the transcriptional activity of the pgtPBREM(-3279G) promoter was ~40% of that of the pgtPBREM(-3279T) promoter. These findings suggested that c.-3279T>G reduced the *UGT1A1* promoter in response to bilirubin.

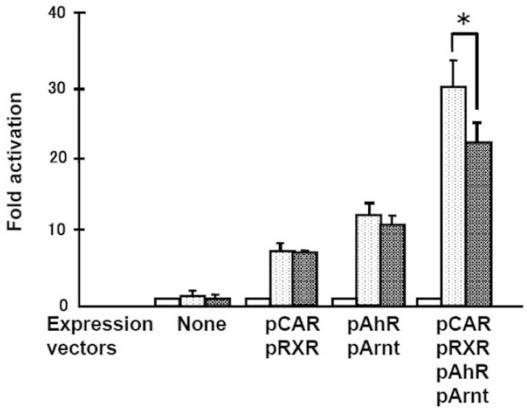


Figure 5. Luciferase reporter assay in COS-7 cells. Transcriptional activity of normal pgtPBREM(-3279T) and mutant pgtPBREM(-3279G) in COS-7 cells was determined in the overexpression status of the nuclear factors, CAR, RXR, AhR, and Arnt. The luciferase activity of pGL2-Basic-miniTK was denoted as 1-fold activation. □, pGL2-Basic-miniTK (Mock); ▨, pgtPBREM (-3279T); and ▩, pgtPBREM (-3279G). *$p < 0.05$.

Second, to determine the effect of the c.-3279T>G mutation on the *UGT1A1* promoter in nonhepatic cell lines, we determined transcriptional activity of pgtPBREM(-3279T) and pgtPBREM(-3279G) in COS-7 cells. Both promoters were synergistically activated by the overexpression of "CAR + RXR," more activated by the overexpression of "AhR + Arnt," and activated the most by the overexpression of all nuclear factors, CAR + RXR and AhR + Arnt. It should be noted that there was no significant difference between the transcriptional activities of the pgtPBREM(-3279T) and pgtPBREM(-3279G) promoters without overexpression of any nuclear factors. In addition, neither CAR + pRXR nor AhR + Arnt caused a significant difference between the transcriptional activities of both promoters. The transcriptional activity of the pgtPBREM(-3279G) promoter became significantly lower than that of the pgtPBREM(-3279T) promoter when all the nuclear factors, CAR, RXR, AhR, and Arnt, were overexpressed (fold activation difference = 7.5, Fig. 5). These results suggested that c.-3279T>G modified the synergistic effects of the nuclear factors.

DISCUSSION

Association of c.-3279T>G in* UGT1A1 *with neonatal jaundice. This is the first report of the allele frequency of c.-3279T>G in Malays. According to Innocenti *et al.,*(10) the allele frequency of c.-3279T>G is 44% in whites and 34% in Asians. Kanai *et al.* (20) reported the allele frequencies of c.-3279T>G in several populations as follows: 26% in Japanese, 33% in Korean, 30% in Chinese, and 35% in German populations. The allele frequency of c.-3279T>G in Malays, 33%, is similar to previous reports from Asian ethnic groups.

In our study, the allele frequency of c.-3279T>G in Malay infants with neonatal jaundice (49%) was more frequent than in those without jaundice (33%). The number of cases with homozygosity for c.-3279T>G was much larger in the jaundice group than in controls. These findings suggest that c.-3279T>G contributes to neonatal jaundice in Malays. How-

ever, Kanai *et al.* (20) reported that there was no significant increase in the allele frequency of c.-3279T>G in infants with neonatal jaundice compared with that in infants without neonatal jaundice in Japanese. The difference between the studies may reflect differences in the genetic or nongenetic background between the ethnic groups.

Modification of the synergistic effects of nuclear factors by c.-3279T>G. Using a luciferase reporter assay with HepG2 cells that expressed AhR, we demonstrated that mutant gtPBREM with c.-3279T>G decreased the transcriptional activity of the *UGT1A1* promoter. In addition, we demonstrated that the c.-3279T>G mutation reduced the responsiveness of gtPBREM to bilirubin (and its possible oxidized products). The detailed mechanism of *UGT1A1* gene activation by bilirubin is not fully understood. Huang *et al.* (21) demonstrated that bilirubin indirectly stimulates CAR accumulation in the nucleus. Recently, Togawa *et al.* (22) indicated that bilirubin also stimulates *UGT1A1 via* binding with AhR. These findings suggested that the *UGT1A1* promoter is activated *via* nuclear factor binding stimulated by bilirubin. Our results also suggest that c.-3279T>G has an inhibitory effect on nuclear factor machinery stimulated by bilirubin.

We showed in the luciferase reporter assay using COS-7 cells that when all the nuclear factors, CAR, RXR, AhR, and Arnt, were overexpressed, there was a significant difference in transcriptional activity between normal and mutant pgtPBREM. The c.-3279T>G mutation did not reduce the transcriptional activity of the luciferase-reporter gene in COS-7 cells without overexpression of nuclear factors. Our findings suggest that c.-3279T>G may modify the synergistic effects of the nuclear factors associated with transcription.

However, it is not clear why the c.-3279T>G mutation reduces transcriptional activity in HepG2 cells without overexpression of nuclear factors, or why the c.-3279T>G mutation does not reduce transcriptional activity in COS-7 cells without overexpression of nuclear factors. Different effects of the same mutation in different cell lines could be explained by the expression conditions of nuclear factors in each cell line.

***Combined effects of c.-3279T>G and other mutations in* UGT1A1.** In our population study, the heterozygous state of the c.-3279T>G mutation appeared in both the jaundice group and controls. In addition, there were many infants with neonatal jaundice who did not carry the c.-3279T>G mutation. These findings strongly suggest that the presence of the c.-3279T>G mutation in only one allele does not cause neonatal jaundice and that other risk factors are also present in neonatal jaundice group.

On the basis of the PHASE analysis, we expected the high prevalence of a *UGT1A1* allele with c.-3279T>G and A(TA)7TAA in the Malay population (Table 2). Homozygosity for c.-3279T>G and A(TA)7TAA may be associated with neonatal jaundice and/or GS. Maruo *et al.* (23) reported that 23 GS patients, including 11 whites and 12 Japanese, were homozygous for c.-3279T>G and A(TA)7TAA. Costa *et al.* (24) also showed that 67 of 74 GS patients were homozygous for c.-3279T>G and A(TA)7TAA. Ferraris *et al.* (25) investigated the role of three *UGT1A1* polymorphisms [A(TA)nTAA, c.-3279T>G, and c.211G>A] in the susceptibility to GS in 53 Italian pediatric subjects compared with 83 unaffected controls. According to Ferraris *et al.*,(25) homozygosity for both A(TA)7TAA and c.-3279G was associated with the highest relative risk estimate (OR = 19.23, 95% CI = 7.34–50.4; $p < 0.001$). All these researchers believed that there is a synergistic effect of c.-3279T>G and A(TA)7TAA and calculated that the association of these two mutations could lower the transcriptional activity of *UGT1A1* to the GS-causing level (23–25).

With regard to mutations in the coding region of *UGT1A1*, we previously reported that the c.211G>A mutation is rare in Malays and Javanese (13,14). However, according to recent reports in the Chinese population in Malaysia and Singapore, the c.211G>A mutation is observed more frequent than Malays and Javanese (26–28). Thus, it can be expected that the frequency of alleles with c.-3279T>G and c.211G>A may be higher in the Chinese (or East Asian) population than in the Malay and Javanese populations. The combined effects of c.-3279T>G and c.211G>A may contribute greatly to the development of GS or neonatal jaundice.

Combined effects of c.-3279T>G in UGT1A1 and mutations in other genes. There are several reports in cases with an inherited *UGT1A1* deficiency and genetic hemolytic disorders such as G6PD deficiency (29,30). They are good examples of combined effects of mutations of *UGT1A1* and other genes. Recently, two other proteins related to neonatal jaundice have been investigated with regard to overflow of nonconjugated bilirubin from the liver; transporter proteins of bilirubin in hepatic cells encoded by the organic anion transporter 2 gene (*OATP2* or *SLC21A6*) (31,32) and the glutathione-S-transferase gene (*GST*) (33,34). However, combined effects of c.-3279T>G in *UGT1A1* and hemolytic disorder-causing genes and combined effects of c.-3279T>G and *OATP2* (or *GST*) mutations remain to be clarified.

Combined effects of c.-3279T>G in UGT1A1 and environmental factors. It is well known that breast feeding increases serum bilirubin levels (35). Maruo *et al.* (36) demonstrated that the c.211G>A mutation is commonly found in infants with unconjugated hyperbilirubinemia because of breast milk feeding in the Japanese population. Similarly, we can expect some combined effects of c.-3279T>G and breast milk feeding on the development of neonatal jaundice, although we could not compare infants fed with breast milk and those fed with formula milk in this study.

Mutations in the UGT1A1 promoter and maturation of UGT1A1 enzyme activity. Neonatal jaundice is associated with the maturation of *UGT1A1* enzyme activity. Kawade and Onishi (37) reported that *UGT1A1* enzyme activity toward bilirubin changes with age. They showed that the activities of the middle fetal, neonatal, and early infantile phases are ~0.1, 0.1–1, and 1–100%, respectively, of the mature-phase values (37). It is likely that the maturation mechanisms of *UGT1A1* activity are associated with the pathway activating the *UGT1A1* promoter. Combination of cis- or trans-acting factors analysis of the *UGT1A1* promoter may offer a clue to understanding the maturation mechanisms of *UGT1A1* activity.

CONCLUSIONS

In the Malay population, c.-3279T>G in *UGT1A1* is a genetic risk factor for neonatal jaundice. Luciferase reporter assay in HepG2 cells indicated that the c.-3279T>G mutation reduces the responsiveness of *UGT1A1* transcription to bilirubin. In addition, luciferase reporter assay in COS-7 cells suggested that c.-3279T>G modifies the synergistic effects of the nuclear factors which are associated with the transcription.

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