Variations in the UDP-glucuronosyltransferase 1A1 gene for the development of unconjugated hyperbilirubinemia in Taiwanese

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Introduction: Results of several studies have indicated that the variation of c.-3279T>G in the UDP-glucuronosyltransferase (*UGT*)1A1 gene could be a further factor for the development of hyperbilirubinemia. However, this variant has not been reported in the Taiwanese population. **Materials & methods:** PCR-restriction fragment length polymorphism was utilized to determine variants at nucleotides -3279 (*60), -53 (*28) and 211 (*6) in the *UGT*1A1 gene for 178 Taiwanese hyperbilirubinemic patients and 200 controls. **Results:** A total of ten and nine diplotypes were observed in the hyperbilirubinemic patients and controls, respectively. Subjects possessing diplotypes of compound haplotypes (*60/*28, *60/*6, *1/*60 plus *1/*28 plus *1/*6); *60/*60; *60/*60 plus 1/*28 and *6/*6 were significantly related to hyperbilirubinemia development, with an odds ratio of 7.83–188.00 ($p = 0.012\sim <0.001$). A subgroup possessing diplotypes of *60/*60 plus *28/*28 were only found in hyperbilirubinemic patients, not in the controls. Bilirubin concentration amongst these patients carrying a diplotype of *60/*60 plus *28/*28 (mean [SD]: 39.2 [10.77] $\mu\text{mol/l}$) was significantly higher than that in the diplotype subgroups of *60/*60 plus *1/*28 (30.4 [4.10] $\mu\text{mol/l}$) and *6/*6 (30.3 [3.08] $\mu\text{mol/l}$) ($p = 0.046$ and 0.034 , respectively). **Conclusions:** The c.-3279T>G variant is a further factor for the development of hyperbilirubinemia. Our results also demonstrate that possessing the *60/*60 plus *28/*28 diplotype in the *UGT*1A1 gene is a determinant of relatively higher bilirubin values amongst hyperbilirubinemic patients.

The cDNA of human UDP-glucuronosyltransferase (*UGT*)1A1 gene was cloned in 1991 [1]. This led to the identification of genetic defects in patients with Crigler–Najjar syndrome type 1 (CN-1, severe hyperbilirubinemia), CN-2 (moderate hyperbilirubinemia) and Gilbert's syndrome (GS; mild hyperbilirubinemia) in 1992, 1993 and 1995, respectively [2–7]. To date, at least 113 variants or mutations of the *UGT*1A1 gene have been reported [101]. GS is an inherited form of mild and chronic unconjugated hyperbilirubinemia without liver disease or overt hemolysis. The results of studies demonstrated that patients with low *UGT*1A1 activity, such as those with GS, may be at an increased risk for chemotherapy toxicity [8]. Therefore, in 2005, the US FDA approved genetic testing for *UGT*1A1*28 (A[TA]₆TAA substituted to A[TA]₇TAA at nucleotide [nt] -53: c.53[TA]₇, rs 34815109) that predict adverse reactions to irinotecan, a potent drug for the treatment of metastatic colorectal cancer [8]. Thus, the molecular study for GS will improve drug safety and impact personalized medicine.

For GS patients, the situation of genetic variation in the *UGT*1A1 gene is discrepant among different ethnic groups. Variation at nt -53 in the

promoter area of the *UGT*1A1 gene has been initially considered to be the sole cause of GS in Caucasian populations, whilst variations in the promoter area, as well as within the coding region of the *UGT*1A1 gene (c.211G>A [p.Gly71Arg], *UGT*1A1*6, rs 4148323) are found in Asian GS patients [9]. Since 2002, results of several studies have indicated that the variation of c.-3279T>G (*UGT*1A1*60, rs 4124874) in the *UGT*1A1 gene promoter region (the phenobarbital-responsive enhancer module) could be a further factor for the development of GS and linkage disequilibrium (LD) existing between -53 and -3279 alleles [10–14]. A combined test for *UGT*1A1 c.-3279T>G and c.-53(TA)₇ polymorphisms was found to be the best item to predict GS in Italian pediatric patients [13], whilst the c.-3279T>G was not observed to be associated with neonatal hyperbilirubinemia in Japanese [15]. In Caucasians, LD was highly significant between sites -3279 and the (TA)₇ polymorphism ($p < 0.0001$) [16]. By contrast, in African-Americans, only a marginal level of significance was observed between (TA)₇ and -3279 ($p = 0.02$) [16]. These results reveal that the c.-3279T>G variant is worth studying for every ethnic group.

Keywords: c.-3279T>G, diplotype, diplotypes of compound haplotypes, hyperbilirubinemia, *UGT*1A1 gene

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Figure 1. Example results of wild-type (TT), heterozygous variation (TG) and homozygous variation (GG) at nucleotide -3279 of the *UGT1A1* gene by PCR-RFLP analysis.

The 20 base pair (bp) fragment is too small to be seen.

M: DNA marker; RFLP: Restriction fragment length polymorphism.

The characteristics of the *UGT1A1* gene in the Taiwanese population were first reported in 2000 by Huang *et al.* [17]. In 2001, a study demonstrated that variations at nt -53 and nt 211 of the *UGT1A1* gene were the major causes for GS in the Taiwanese population [18]. Results of our studies showed that variation in the *UGT1A1* gene was the major hereditary factor for the development of GS in the Taiwanese population, whilst glucose-6-phosphate dehydrogenase (G6PD) deficiency and variations in the organic anion transporter polypeptide 2 gene were two additive factors [19,20]. Very recently, we reported that combined *UGT1A1* and *UGT1A7* variant alleles were associated with increased risk of GS [21]. However, the -3279 variant of the *UGT1A1* gene has not yet been reported in the Taiwanese population. In the present study, in order to evaluate the effect of the c.-3279T>G upon the development and bilirubin values of unconjugated hyperbilirubinemia, we focused hereditary research on the *UGT1A1* gene and investigated the three variants of this gene for newly-enrolled Taiwanese hyperbilirubinemic patients and gender-/age-matched controls.

Materials & methods

Patients & controls

The diagnosis of unconjugated hyperbilirubinemia was based on the criteria of serum total bilirubin $\geq 27.4 \mu\text{mol/L}$, unconjugated bilirubin/total bilirubin $\geq 80\%$, normal liver function tests, no overt signs of hemolysis over a period greater than 4 months [19–21], and normal G6PD activity in erythrocytes by using the quantitative enzyme-coupled method as described previously [19,20]. Bilirubin values were determined in the morning following ≥ 8 h of fasting and measurements were carried out at

least twice within 6 months. Bias of the bilirubin value for every individual was less than 10% and the higher (highest) value was taken into calculation in this study. A total of 124 unrelated Taiwanese men (aged 21–60 years, mean [SD]: 37.4 [10.0] years) and 54 unrelated Taiwanese, age-matched women (aged 21–59 years, 36.1 [9.0] years) with hyperbilirubinemia, identified from the gastroenterology clinic at the Cathay General Hospital, Taipei, Taiwan, were enrolled. Another 140 healthy Taiwanese men (aged 22–62 years, 38.5 [10.3] years) and 60 age-matched Taiwanese women (aged 22–56 years, 36.7 [9.1] years), with normal liver and hematological test results and serum total bilirubin $<25.7 \mu\text{mol/L}$, were selected from subjects having physical examinations at the same hospital as the control group. All subjects provided their informed consent to participate in this study, which was approved by the ethical committee of the Cathay General Hospital, Taipei, Taiwan.

Determination of *UGT1A1* gene variants

Genomic DNA was isolated from whole blood cells using a blood DNA isolation kit (Maxim Biotech Inc., CA, USA). The SNPs of c.-53(TA)₇ in the promoter area and c.211G>A within the coding region of the *UGT1A1* gene were determined by PCR-restriction fragment length polymorphism (PCR-RFLP) as described previously [20,21] for all the study subjects. The DNA of the two known SNPs of the *UGT1A1* gene, which were previously identified by DNA sequencing method [17], were run as positive controls in the performance of each genotyping assay. In addition, PCR-RFLP was utilized to detect *UGT1A1* -3279 polymorphism by using the natural or mutagenic primers and the restriction enzyme *DraI* (Table 1 & Figure 1). The DNAs of the wild-type, heterozygous variation and homozygous variation at nt -3279, which were determined by DNA sequencing with 5'-CAC-CAGAACAACCTTCTGAG-3' as forward primer and 5'-CTGTCCCTTCTGAAT-CATTG-3' as reverse primer [10,16], were run as positive controls in each performance of the PCR-RFLP assays.

Statistic analysis

Two sided t-test was used to compare bilirubin values between male and female subjects to decide whether or not further study would be separately performed for both genders. The pairwise D' and r^2 values for LD for the variation of

Table 1. Natural or mutagenic primers, restriction enzyme and *UGT1A1* -3279 polymorphism.

Position	Primer	Sequence	Restriction enzyme	Result, base pair
-3279	U-3279F	5'-CACCAGAACAACTTCTGAG-3'	<i>Dra I</i>	T 121 + 20
T>G	U-3279R	5'-GTTCTCAAATTGCTTTGTTA-3'		G 141

*Section highlighted in bold indicates mutagenic site.

nt -3279 with the (TA)₇ in GS patients were calculated by using a LD plotter [102]. Then the study subjects were divided into several diplotypes according to combinations of the three SNPs. Simple logistic regression was utilized to calculate odds ratios (ORs; of diplotypes carrying SNPs to the wild-type) and the 95% confidence intervals (CIs) for hyperbilirubinemic patients' diplotypes. Analysis of variance (ANOVA) was used to compare bilirubin values amongst the various diplotypes. When the result of ANOVA was statistically significant, multiple comparisons were performed using the Scheffé *post-hoc* method. A p-value less than 0.05 was defined as statistically significant. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 10.0 software (SPSS for Windows, Inc., IL, USA).

Results

Distribution of *UGT1A1* diplotypes

The lowest and highest bilirubin values were 8.6 µmol/l (0.5 mg/dl) and 68.4 µmol/l (4.0 mg/dl), respectively. These were found in one of the female controls and one of the male hyperbilirubinemic patients, respectively. There was no difference in the serum bilirubin values between male and female hyperbilirubinemic patients (mean [SD]: 34.4 [8.21] µmol/l versus 32.5 [7.01] µmol/l, p= 0.145). Bilirubin values also did not differ between male and female controls (15.8 [4.10] µmol/l versus 14.9 [3.76] µmol/l, p= 0.151). Thus, for further study, the men and women were pooled for hyperbilirubinemic patients and controls.

Results of the PCR-RFLP showed that both known haplotypes (*28 and *6) and the newly

Figure 2. Distribution of serum bilirubin levels among the subgroups of hyperbilirubinemic patients carrying different *UGT1A1* diplotypes.

Error bars represent 95% CI, the numbers of each diplotype are listed in Table 2.
CI: Confidence interval.

Table 2. Diplotypes of the *UGT1A1* gene in patients with unconjugated hyperbilirubinemia and controls.

No.	Diplotype	Patients [†] (n = 178)	Controls [§]			
			(n = 200)	OR	95% CI	p-value
1	*1/*1	2	47	1.00		
2	*1/*60	8	44	4.27	0.86–21.23	0.076
3	*1/*6	2	30	1.57	0.21–11.72	0.662
4	*60/*28	9	27	7.83	1.58–38.94	0.012
5	*60/*6	20	21	22.38	4.79–104.60	<0.001
6	*1/*60, *1/*28, *1/*6	48	6	188.00	36.10–978.98	<0.001
7	*60/*60	4	5	18.80	2.72–129.73	0.003
8	*60/*60, *1/*28	18	16	26.44	5.52–126.71	<0.001
9	*6/*6	19	4	111.63	18.84–661.29	<0.001
10	*60/*60, *28/*28	48	0			

*1: wild-type; *60: T changed to G at nucleotide -3279; *28: A(TA)₆TAA changed to A(TA)₇TAA at nucleotide -53; *6: G changed to A at nucleotide 211.

[†]Diplotype patients. Linkage disequilibrium between c.-3279 T>G and c.-53(TA)₇ alleles in the patients: D' = 1, r² = 0.54, p < 0.001.

[§]Controls. For the control group, the genotype distributions for nucleotides -3279 and 211 were in Hardy–Weinberg equilibrium.

CI: Confidence interval; OR: Odds ratio.

detected haplotype (*60) in the *UGT1A1* gene were found in both hyperbilirubinemic patients and controls (Table 2). The results of the LD plot showed that D' and r² values for the association of the variation of c.-3279T>G with the c.-53(TA)₇ in hyperbilirubinemic patients were 1 and 0.54, respectively (p < 0.001). For the control group, the genotype distributions for nt -3279 and nt 211 were in Hardy–Weinberg equilibrium, whilst the homozygous variation (A[TA]₇TAA/A[TA]₇TAA) was absent for nt -53.

As shown in Table 2, there were ten and nine diplotypes observed in the hyperbilirubinemic patients and the controls, respectively. The diplotypes of compound haplotypes (*60/*28, *60/*6, *1/*60 plus *1/*28 plus *1/*6; *60/*60, *60/*60 plus *1/*28 and *6/*6 (diplotypes no. 4–9) were significantly related to hyperbilirubinemia development (OR: 7.83–188.00, p = 0.012 to <0.001). Only hyperbilirubinemic patients were found to possess a diplotype of *60/*60 plus *28/*28 (diplotype no. 10), not the controls. On the other hand, ORs for the diplotypes of *1/*60 and *1/*6 (diplotypes no. 2 and 3) did not prove to be meaningful.

Contribution of c.-3279T>G to hyperbilirubinemia

Amongst the six diplotypes significantly associated with hyperbilirubinemia development (*60/*28, *60/*6, *1/*60 plus *1/*28 plus *1/*6, *60/*60, *60/*60 plus *1/*28 and *6/*6), except for those with *6/*6, the c.-3279T>G variant

was observed in every hyperbilirubinemic patient. Moreover, the diplotype of compound homozygotes consisted of *60/*60 plus *28/*28. Subjects possessing the c.-3279T>G variant accounted for 87.1% (155/178) of the 178 hyperbilirubinemic patients. The c.-3279T>G is the dominant variant of the three SNPs found in hyperbilirubinemic patients.

Bilirubin concentrations

The results of ANOVA demonstrated that bilirubin values among the ten haplotypes of hyperbilirubinemic patients were not equal (F = 4.497; p < 0.001). Table 3 & Figure 2 show that, amongst hyperbilirubinemic patients, the serum bilirubin level (39.2 [10.77] μmol/l) in the subgroup carrying a diplotype of *60/*60 plus *28/*28 (diplotype no. 10) was significantly higher than that (30.4 [4.10] μmol/l and 30.3 [3.08] μmol/l, respectively) in the diplotype subgroups of *60/*60 plus *1/*28 (diplotype no. 8) and *6/*6 (diplotype no. 9) (p = 0.046 and 0.034, respectively), whilst there was no difference in bilirubin values between the other two-subgroup comparisons.

For the comparison of bilirubin values among the nine diplotypes of controls, the results of ANOVA showed those were not equal (F = 4.594; p < 0.001). Table 3 indicates that the serum bilirubin level (13.5 [2.74] μmol/l) in the subgroup carrying a diplotype of *1/*1 (diplotype no. 1) was significantly lower than that (17.6 [3.76] μmol/l and 20.5 [2.08] μmol/l, respectively) in the diplotype

Table 3. Bilirubin values in the subjects carrying different diplotypes of the *UGT1A1* gene.

No.	Diplotype	Bilirubin value		Bilirubin value	
		Patients	p-value	Controls	p-value
1	*1/*1	27.4 (0.00)	<0.001	13.5 (2.74) [†]	<0.001
2	*1/*60	28.2 (0.86)	(F = 4.497)	15.0 (3.76)	(F = 4.594)
3	*1/*6	34.2 (7.18)		16.1 (4.45)	
4	*60/*28	34.4 (6.50)		15.6 (3.59)	
5	*60/*6	33.3 (6.16)		17.6 (3.76) [§]	
6	*1/*60, *1/*28, *1/*6	33.2 (6.50)		20.5 (3.08) [§]	
7	*60/*60	28.2 (1.71)		17.8 (4.62)	
8	*60/*60, *1/*28	30.4 (4.10) [¶]		15.6 (4.10)	
9	*6/*6	30.3 (3.08) [¶]		18.0 (3.25)	
10	*60/*60, *28/*28	39.2 (10.77) [#]			

[†]p = 0.023 (no. 1 versus No. 5) by Scheffé post-hoc test. The figures in the parentheses represent standard deviations of bilirubin values.

[§]p = 0.017 (no. 1 versus No. 6) by Scheffé post-hoc test. The figures in the parentheses represent standard deviations of bilirubin values.

[¶]p = 0.046 (no. 8 versus no. 10) by Scheffé post-hoc test.

[#]p = 0.034 (no. 9 versus no. 10) by Scheffé post-hoc test.

subgroups of *60/*6 (diplotype no. 5) and *1/*60 plus *1/*28 plus *1/*6 (diplotype no. 6) (p = 0.023 and 0.017, respectively). There was no difference in bilirubin values between the other two subgroup comparisons.

Discussion

This is the first time a study had been performed for determining the variant c.-3279T>G of the *UGT1A1* gene for Taiwanese. In this study, all the hyperbilirubinemic patients carrying *1/*28 were coinherited with *1/*60 or *60/*60. This is in agreement with the study of European Caucasian GS patients [12]. Our results also demonstrated that all the hyperbilirubinemic patients carrying *28/*28 were coinherited with the *60/*60. Such a status of compound homozygotes was absent in our controls, consistent with that reported in Caucasian and Japanese GS patients [11]. Our results demonstrated that *6/*6 was another main variant for hyperbilirubinemia.

Our results showed that subjects possessing diplotypes of compound heterozygotes, homozygotes and compound homozygotes in the *UGT1A1* gene were the indicators of hyperbilirubinemia. Those diplotypes mainly consisted of *1/*60 plus *1/*28 plus *1/*6, *60/*60 plus *1/*28, *6/*6 and *60/*60 plus *28/*28. Our results demonstrated that subjects with diplotype of compound homozygotes in the *UGT1A1* gene (diplotype no. 10) will develop hyperbilirubinemia; this is true for Taiwanese adults. As shown in Table 2 of this study, ORs of diplotypes no. 5–9 were higher than 18.00,

whilst that of diplotype no. 4 was 7.83. Sample sizes of the hyperbilirubinemic patients and the controls for diplotype no. 4 were 9 and 27, respectively, approximately fitted to the sample sizes necessary in order to detect the significant association (power = 80%, two sided $\alpha = 0.05$) [22].

We demonstrated that c.-3279T>G was a further contributor for Taiwanese with hyperbilirubinemia. The results of a study showed that the genetic diagnosis of GS for Japanese and Caucasians could be easily established by genotyping of the c.-3279T>G locus [11], whilst those of another two studies indicated that determination for genotype c.-3279T>G instead of the TATAA-box of the *UGT1A1* gene promoter may be misleading for Caucasians [12,14]. The contradictory results were depended on the degree of LD status between these two alleles [14]. The present study demonstrated that LD between these two alleles in Taiwanese hyperbilirubinemic patients was complete ($D' = 1$) but not perfect ($r^2 = 0.54$), consistent with the findings in the latter two studies mentioned above [12,14]. Our results showed that, for hyperbilirubinemia in Taiwanese, linkage between c.-3279T>G and c.-53(TA)₇ variations in the *UGT1A1* gene was not essential for pathogenesis, whilst c.211G>A was an independent contributor. The result is coincident with the finding reported by Minami *et al.* that the homozygotes and double heterozygotes of *6 and *28 were significantly associated with severe neutropenia after irinotecan treatment [23].

In different experiments, c.-3279T>G polymorphism reportedly decreased the transcriptional activity of the *UGT1A1* gene to 62% of normal [10] and the (TA)₇ allele at nt -53 decreased it to only 33% or 60–80% of normal [6,24]. The *UGT1A1* enzyme activities of the individuals featuring *6/*6 and *1/*6 in the *UGT1A1* gene were 32.2% and 60.2% of normal, respectively [25]. The decreased *UGT1A1* enzyme functionality was assumed to cause hyperbilirubinemia in the subjects carrying those variants. We found that bilirubin levels in the hyperbilirubinemic patients carrying *60/*60 plus *28/*28 were significantly higher than those of the subjects bearing *60/*60 plus *1/*28, consistent with that observed in Caucasians [12]. Moreover, our results demonstrated for the first time that the hyperbilirubinemic patients carrying *60/*60 plus *28/*28 had higher bilirubin levels than the subgroup possessing *6/*6. This may be attributable to the fact that hyperbilirubinemic patients carrying such compound homozygotes have lower enzyme function than in those bearing the other *UGT1A1* diplotypes. As shown in Table 3 & Figure 2, hyperbilirubinemic patients carrying *60/*60 plus *28/*28 (diplotype no. 10) seem to have higher bilirubin values than those possessing wild-type (diplotype no. 1), *1/*60 (diplotype no. 2) and *60/*60 (diplotype no. 7). However, statistical results did not prove such differences. This may be attributable to the fact that the sample size of those three diplotypes is too small (2, 8 and 4, respectively).

Although the prevalence of *28/*28 is approximately 20% in Caucasians, none or very little (<1%) of this diplotype was found in the Taiwanese control group [20,21] and present study. Since in Taiwanese all the subjects carrying *28/*28 were coinherited with *60/*60, the *UGT1A1* function of such a compound

homozygotes may be low enough to cause hyperbilirubinemia. This is the reason, at least partially, that *28/*28 is seldom found in the controls.

In conclusion, polymorphism c.-3279T>G in the *UGT1A1* gene is a further factor for the development of hyperbilirubinemia. Our results also demonstrate that possessing *60/*60 plus *28/*28 in the *UGT1A1* gene is a determinant of relatively higher bilirubin values amongst patients with hyperbilirubinemia.

Future perspective

In addition to *UGT1A1**28, *UGT1A1**60 and *UGT1A1**6 should be considered in genetic testing for the *UGT1A1* gene for drug safety in Taiwanese. Moreover, variation status in the *UGT1A1* gene, such as compound homozygotes (*60/*60 plus *28/*28) and homozygote (*6/*6), may be utilized as markers to predict chemotherapy-induced severe toxicity for Taiwanese.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The results of studies demonstrated that patients with low UDP-glucuronosyltransferase (*UGT*)1A1 activity, such as those with Gilbert's syndrome (GS), may be at an increased risk for chemotherapy toxicity. The molecular study for GS will improve drug safety and impact on personalized medicine.
- Several studies have reported that the variation of c.-3279T>G in the *UGT1A1* gene promoter region could be a further factor for the development of GS.
- In the present study, the c.-3279T>G variation is determined for Taiwanese for the first time.
- The results of this study show that the c.-3279T>G is a further factor for the development of hyperbilirubinemia in Taiwanese, whilst c.211G>A is an independent contributor.
- Our results also demonstrate that possessing compound homozygotes (*60/*60 plus *28/*28) in the *UGT1A1* gene is a determinant of relatively higher bilirubin values amongst hyperbilirubinemic patients.

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