

## Effects of variant UDP-glucuronosyltransferase 1A1 gene, glucose-6-phosphate dehydrogenase deficiency and thalassemia on cholelithiasis

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**CONCLUSION:** Our results show that the homozygous variation in the *UGT1A1* gene is a risk factor for the development of cholelithiasis in Taiwan Chinese.

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**Key words:** *UGT1A1* gene; G6PD deficiency; Thalassemia; Cholelithiasis

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### Abstract

**AIM:** To test the hypothesis that the variant UDP-glucuronosyltransferase 1A1 (*UGT1A1*) gene, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and thalassemia influence bilirubin metabolism and play a role in the development of cholelithiasis.

**METHODS:** A total of 372 Taiwan Chinese with cholelithiasis who had undergone cholecystectomy and 293 healthy individuals were divided into case and control groups, respectively. PCR and restriction fragment length polymorphism were used to analyze the promoter area and nucleotides 211, 686, 1 091, and 1 456 of the *UGT1A1* gene for all subjects and the gene variants for thalassemia and G6PD deficiency.

**RESULTS:** Variation frequencies for the cholelithiasis patients were 16.1%, 25.8%, 5.4%, and 4.3% for A(TA)<sub>6</sub>TAA/A(TA)<sub>7</sub>TAA (6/7), heterozygosity within the coding region, compound heterozygosity, and homozygosity of the *UGT1A1* gene, respectively. Comparing the case and control groups, a statistically significant difference in frequency was demonstrated for the homozygous variation of the *UGT1A1* gene ( $P = 0.012$ ,  $\chi^2$  test), but not for the other variations. Further, no difference was demonstrated in a between-group comparison of the incidence of G6PD deficiency and thalassemia (2.7% vs 2.4% and 5.1% vs 5.1%, respectively). The bilirubin levels for the cholelithiasis patients with the homozygous variant-*UGT1A1* gene were significantly different from the control analog ( $18.0 \pm 6.5$  and  $12.7 \pm 2.9$   $\mu\text{mol/L}$ , respectively;  $P < 0.001$ , Student's *t* test).

### INTRODUCTION

The life span of erythrocytes in individuals with chronic hemolytic disease is short relative to normal subjects. The accelerated red-cell turnover produces more bilirubin and is probably a risk factor for the development of gallstones. Cholelithiasis has been detected in 38% of subjects with chronic hemolytic anemia due to glucose-6-phosphate dehydrogenase (G6PD) deficiency<sup>[1]</sup>. It has been demonstrated that G6PD-deficient subjects without chronic hemolytic anemia also have a higher frequency of cholelithiasis development than normal individuals<sup>[1]</sup>. Because the enzyme UDP-glucuronosyltransferase 1A1 (*UGT1A1*) catalyzes unconjugated bilirubin into the diglucuronidated and conjugated forms, *UGT1A1* defects decrease the elimination of bilirubin<sup>[2]</sup>. Genetic variation in the *UGT1A1* gene promoter, A(TA)<sub>7</sub>TAA, instead of A(TA)<sub>6</sub>TAA influences bilirubin levels causing hyperbilirubinemia, which is associated with Gilbert's syndrome in Caucasians<sup>[2]</sup>. Further, it has been determined that *UGT1A1* gene expression is a major modifying factor for bilirubin levels in heterozygous  $\beta$ -thalassemia<sup>[3]</sup> and that cholelithiasis is one of the manifestations of thalassemia<sup>[4]</sup>.

In a previous study of the *UGT1A1* gene in Taiwan Chinese<sup>[5]</sup>, we found that occurrence of the A(TA)<sub>7</sub>TAA allele in the promoter area was relatively rare (14.3% vs 40%) and that the variation rate within the coding region was much higher (29.3% vs 0.1%) relative to Caucasians<sup>[2]</sup>, respectively. The aim of this study was to analyze variations in the promoter area and coding region in the *UGT1A1* gene in Taiwan Chinese patients with cholelithiasis. The incidence and genetic expression of G6PD deficiency in

these subjects was also investigated. Additionally, we studied the genetic alteration of thalassemia. Variations in the *UGT1A1*, *G6PD* and thalassemia genes were compared with those of normal subjects. To our knowledge, this is the first report elucidating the relationship between these three genes and cholelithiasis.

## MATERIALS AND METHODS

### Study subjects, healthy controls, and laboratory tests

The study subjects (157 males and 215 females) were selected from Taiwan Chinese adults who had undergone cholecystectomy for symptomatic gallstone. Healthy controls (135 males and 158 females) were selected from adults who had undergone physical examination at Cathay General Hospital as previously described<sup>[5]</sup>. The control subjects were investigated for cholelithiasis by abdominal ultrasonography (model SSD-1700, Aloka Co., Tokyo, Japan) and no change in abdomen was determined with respect to gallstones. Blood samples were collected from all subjects in the morning after 10-12 h of fasting. An automatic cell-counter (NE 9000, Sysmex Co., Kobe, Japan) was used for hematology testing, and erythrocyte G6PD activity was quantitatively measured using the enzyme-coupled method<sup>[6]</sup>. G6PD deficiency was confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)<sup>[7]</sup>. The 15 single-nucleotide mutations, restriction enzymes, and restriction-fragment results are listed in Table 1, with priority arranged in descending order of mutation frequency of our Taiwan Chinese sample population<sup>[7]</sup>. The promoter area and nucleotides 211, 686, 1 091, and 1 456 of the *UGT1A1* gene were amplified by PCR and analyzed using RFLP<sup>[8]</sup>. The restriction enzymes and restriction-fragment results are listed in Table 2. Mean corpuscular volume  $\leq 80$  fL, the gene variants for  $\alpha$ - and  $\beta$ -thalassemia were identified using PCR-RFLP<sup>[9,10]</sup>.

### Comparison of bilirubin values

Serum bilirubin levels were measured using an autoanalyzer (model 747, Hitachi Co., Tokyo, Japan). The bilirubin values

in cholelithiasis patients with statistically significant differences in gene frequency relative to controls were obtained from the records made 2-3 mo after cholecystectomy and compared with that in the control group.

### Statistical analysis

The  $\chi^2$  test, with or without Yate's correction, and Student's *t* test were used to analyze the data as appropriate. A *P* value less than 0.05 was defined as statistically significant.

## RESULTS

G6PD deficiency was confirmed for 4 of the 157 males (2.5%) and 6 of the 215 females (2.8%) cholelithiasis patients, with respective gender distribution as follows: hemizygous 493, 871, 1 024, and 1 388 ( $n = 1$  each), and heterozygous 1 376 ( $n = 5$ ) and heterozygous 1 388 ( $n = 1$ ) (data not shown in Tables). There was no significant between-gender difference in incidence of G6PD deficiency. Analysis of pooled data showed an overall incidence of G6PD deficiency of 2.7% (10/372) for the cholelithiasis group. The frequencies of the *UGT1A1* genotypes are presented in Table 3. Less than half (48.4%) of the cholelithiasis patients carried the wild genotype. The variation frequencies for A(TA)<sub>6</sub>TAA/A(TA)<sub>7</sub>TAA (6/7), heterozygosity within the coding region, compound heterozygosity, and homozygosity were 16.1%, 25.8%, 5.4%, and 4.3%, respectively. The incidence of thalassemia in the cholelithiasis patients was 5.1% (19/372) [heterozygous  $\alpha$ -thalassemia 2.7% ( $n = 10$ ); heterozygous  $\beta$ -thalassemia 1.6% ( $n = 6$ ); homozygous  $\beta$ -thalassemia 0.53% ( $n = 2$ ); and heterozygous  $\alpha$ -thalassemia plus heterozygous  $\beta$ -thalassemia 0.27% ( $n = 1$ )] (detailed data not shown in Tables). Analysis for the combination of G6PD deficiency, homozygous variation in the *UGT1A1* gene, and presence of thalassemia revealed that two cholelithiasis patients carried the 211 G $\rightarrow$ A/211 G $\rightarrow$ A *UGT1A1* and the heterozygous  $\alpha$ -thalassemia genes (data not shown in Tables).

Clinical data for the cholelithiasis patients and healthy controls are compared in Table 3. There was no difference in gender distribution, neither in the mean of age, comparing the two groups. A significant difference was demonstrated comparing the frequency of homozygous variation (7/7, 211 G $\rightarrow$ A/211 G $\rightarrow$ A, and 686 C $\rightarrow$ A plus 7/7) of the *UGT1A1* gene for the case and control groups ( $P = 0.012$ ,  $\chi^2$  test), while those for the other three variation types were not statistically meaningful. There was no significant between-group difference in the incidence of G6PD deficiency and thalassemia (2.7% *vs* 2.4% and 5.1% *vs* 5.1%, respectively). Nine (56.3%) of the 16 cholelithiasis patients

**Table 1** Fifteen single nucleotide mutations of G6PD deficiency in Taiwan Chinese

Mutation site	Restriction enzyme	Restriction fragment (bp)	
		Normal	Mutant
1 376 G $\rightarrow$ T	<i>Afl</i> I	214	194, 20
1 388 G $\rightarrow$ A	<i>Nde</i> I	227	206, 21
493 A $\rightarrow$ G	<i>Ava</i> II	120, 11	87, 33, 11
1 024 C $\rightarrow$ T	<i>Mbo</i> II	187	150, 37
95 A $\rightarrow$ G	<i>Mlu</i> I	198	174, 24
392 G $\rightarrow$ T	<i>Bst</i> EII	188, 15	203
871 G $\rightarrow$ A	<i>Bgl</i> III	118	94, 24
487 G $\rightarrow$ A	<i>Hind</i> III	104	82, 22
519 C $\rightarrow$ G	<i>Sty</i> I	300	189, 111
835 A $\rightarrow$ T	<i>Taq</i> I	185	164, 21
1 360 C $\rightarrow$ T	<i>Hha</i> I	142, 45, 27	187, 27
592 C $\rightarrow$ T	<i>Pst</i> I	157, 83	157, 63, 20
517 T $\rightarrow$ C	<i>Xho</i> I	134	114, 20
1 004 C $\rightarrow$ A	<i>Hha</i> I	49, 20	69
1 387 C $\rightarrow$ T	<i>Hha</i> I	105, 22	127

**Table 2** Variations of the *UGT1A1* gene

Variation site	Restriction enzyme	Restriction fragment (bp)	
		Normal	Variant
211 G $\rightarrow$ A	<i>Ava</i> II	128, 18	146
686 C $\rightarrow$ A	<i>Bsr</i> I	374, 51	242, 132, 51
1 091 C $\rightarrow$ T	<i>Bcl</i> II	209	190, 19
1 456 T $\rightarrow$ G	<i>Ava</i> II	270	197, 73
Promoter		77	79

**Table 3** Comparison of clinical data for cholelithiasis patients and healthy controls

	Cholelithiasis patients (n = 372)		Control group (n = 293)		P ( $\chi^2$ test) (for age by student's t test)
	n	%	n	%	
Gender (M/F)	157/215		135/158		NS
Age (yr, mean $\pm$ SD)	53.0 $\pm$ 14.9		51.5 $\pm$ 10.6		NS
<i>UGT1A1</i> genotype					
Wild type	180	48.4	143	48.8	NS
Heterozygous variation in promoter area (6/7)	60	16.1	60	20.5	NS
Heterozygous variation within coding region	96	25.8	64	21.8	NS
211 G $\rightarrow$ A/normal	89		57		
1 091 C $\rightarrow$ T/normal	6		7		
1 456 T $\rightarrow$ G/normal	1				
Compound heterozygous variation	20	5.4	23	7.9	NS
6/7, 211 G $\rightarrow$ A/normal	10		4		
6/7, 686 C $\rightarrow$ A/normal	8		14		
6/7, 1 091 C $\rightarrow$ T/normal			1		
6/7, 211 G $\rightarrow$ A/normal, 686 C $\rightarrow$ A/normal			1		
6/7, 686 C $\rightarrow$ A/normal, 1 091 C $\rightarrow$ T/normal	1		1		
211 G $\rightarrow$ A/normal, 1 091 C $\rightarrow$ T/normal	1		1		
Homozygous variation	16	4.3	3	1.0	0.012
7/7	5				
211 G $\rightarrow$ A/G $\rightarrow$ A	4		3		
7/7, 686 C $\rightarrow$ A/normal	7		0		
G6PD deficiency	10	2.7	7	2.4	NS
Thalassemia	19	5.1	15	5.1	NS

6/7: A(TA)<sub>6</sub>TAA/A(TA)<sub>7</sub>TAA, 7/7: A(TA)<sub>7</sub>TAA/A(TA)<sub>7</sub>TAA in the promoter area of *UGT1A1* gene.

with the homozygous variant-*UGT1A1* gene had bilirubin levels  $\geq 17.1$   $\mu\text{mol/L}$  (upper limit of normal reference value) with significant difference between means in comparing the 16 patients with the controls [18.0 $\pm$ 6.5  $\mu\text{mol/L}$  (range 6.8-32.5  $\mu\text{mol/L}$ ) and 12.7 $\pm$ 2.9  $\mu\text{mol/L}$  (range 6.8-17.1  $\mu\text{mol/L}$ ), respectively;  $P < 0.001$ , Student's *t* test] (data not shown in Tables).

## DISCUSSION

No gender difference has been demonstrated in the incidence of variant *UGT1A1* gene and thalassemia, while G6PD deficiency is X-linkage transmitted<sup>[11]</sup>. In this study, as the male/female ratio was approximately equal for the case and control groups, the genders were pooled for analysis. More than 50% of the members of both groups had at least one heterozygous variation in the *UGT1A1* gene, while the frequency of homozygous variation differed between groups. The 686 C $\rightarrow$ A variation was associated with the 6/7 or 7/7 variation for both study subjects and the controls, as observed in our previous studies<sup>[5,7,8,12,13]</sup>. In the present investigation, however, the 7/7 and 686 C $\rightarrow$ A plus 7/7 variations were found only in the case group (Table 3). Since bilirubin may be affected by cholelithiasis status, bilirubin values were obtained for cholelithiasis patients with the homozygous variant-*UGT1A1* gene from the records which were made 2-3 mo post cholecystectomy. *UGT1A1* enzyme activity was found to be 18-33% of normal in individuals with the 7/7 variation of the *UGT1A1* gene<sup>[2]</sup>. In another *in vitro* gene-expression study<sup>[14]</sup>, subjects bearing the homozygous 211 G $\rightarrow$ A variant-*UGT1A1* gene were demonstrated to have 32.2% of the *UGT1A1* enzyme activity of normal analogs. Moreover, coinheritance of

heterozygous  $\alpha$ -thalassemia gene was observed in two of our four cholelithiasis patients who carried the homozygous 211 G $\rightarrow$ A variation. These findings may account for the significant difference in serum bilirubin levels in cholelithiasis patients with the homozygous variant-*UGT1A1* gene (7/7, 211 G $\rightarrow$ A/211 G $\rightarrow$ A, and 686 C $\rightarrow$ A plus 7/7) related to controls.

Although it has been determined that G6PD deficiency and thalassemia are risk factors for cholelithiasis development in Caucasians<sup>[1,4]</sup>, these associations were not observed in our sample, with no between-group difference in incidences of these two factors were noted. This discrepancy may be due to the ethnic differences between the sample populations. Co-inheritance of the homozygous variant-*UGT1A1* gene and G6PD deficiency may lead to pronounced hyperbilirubinemia in adults<sup>[13]</sup>, and co-inheritance of Gilbert's syndrome and hereditary spherocytosis may increase the risk for developing gallstones<sup>[15]</sup>. However, these co-inheritances were not noted in this study. Structural analysis of gallstones from a sample of Taiwan Chinese patients revealed that a relatively small proportion of the gallstones were composed of bilirubin (pure cholesterol 35%, combination 12%, mixed 17%, black 25%, calcium bilirubinate 8%, and unclassified 3%)<sup>[16]</sup>. It is not surprising, therefore, that only 4.3% of our cholelithiasis patients carried the homozygous variant-*UGT1A1* gene, which leads to the bilirubin accumulation and is a risk factor for cholelithiasis. We suggest that investigation of cholelithiasis status and carriage of variant *UGT1A1* gene for specific subpopulations, such as G6PD-deficient or thalassemic Taiwan Chinese, is a superior alternative in genetic research for gallstones. A research program of this type is ongoing at the Cathay General Hospital.

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