UGT1A1 variation and gallstone formation in sickle cell disease


Pigment gallstones are a common clinical complication of sickle cell (SS) disease. Genetic variation in the promoter of uridine diphosphate (UDP)–glucuronosyltransferase 1A1 (UGT1A1) underlies Gilbert syndrome, a chronic form of unconjugated hyperbilirubinemia, and appears to be a risk factor for gallstone formation. We investigated the association between UGT1A1 (TA)n promoter region was sequenced in 541 SS disease subjects and 111 healthy controls (control sample). Indirect bilirubin levels for (TA)4/(TA)4 and (TA)6/(TA)6 genotypes were elevated compared with (TA)4/(TA)4 (clinic sample, \( P < 10^{-5} \); cohort sample, \( P < 10^{-3} \)). The (TA)4/(TA)4 genotype was also associated with symptomatic presentation and gallstones in the clinic sample (odds ratio \([\text{OR}] = 11.3; P = 7.0 \times 10^{-4}\) but not in the younger cohort sample. These unexpected findings indicate that the temporal evolution of symptomatic gallstones may involve factors other than the bilirubin level. Although further studies of the pathogenesis of gallstones in SS disease are required, the (TA)4/(TA)4 genotype may be a risk factor for symptomatic gallstones in older people with SS disease.

Introduction

Bilirubin catabolism is the final step in the breakdown of heme from hemoglobin and hemoprotein turnover. The primary bilirubin catabolizing hepatic enzyme uridine diphosphate (UDP)–glucuronosyltransferase 1A1 (UGT1A1) mediates the conjugation of bilirubin into a water-soluble form that is excreted in bile. A simple dinucleotide repeat polymorphism, (TA)n, in the TATA box of the UGT1A1 gene is associated with reduced expression of the hepatic enzyme and results in the chronic unconjugated hyperbilirubinemia that occurs in Gilbert syndrome.

The coinheritance of Gilbert syndrome with disorders that increase the turnover of red blood cells, or their precursors, has been reported to elevate bilirubin levels in \( \beta \)-thalassemia, G6PD deficiency, and hydroxyurea therapy response in sickle cell (SS) disease. Associations among UGT1A1 promoter variation, high indirect bilirubin levels, and gallstone formation have also been reported in small numbers of subjects with \( \beta \)-thalassemia, hemoglobin \( E/\beta \)-thalassemia, hereditary spherocytosis, and SS disease.

Here we describe an analysis of UGT1A1 promoter polymorphism, bilirubin levels, and gallstone development in a subset of subjects enrolled in the Jamaican Sickle Cell Cohort Study and similar analyses conducted on a series of subjects recruited from the Sickle Cell Clinic of the Sickle Cell Unit, Tropical Medicine Research Institute (TMRI), at the University of the West Indies, Mona, Kingston, Jamaica. This study provides a long-term assessment of genetic factors that influence bilirubin levels and the evolution of gallbladder disease.

Subjects, materials, and methods

Study site

The Sickle Cell Clinic was established in 1965 to provide specialized medical care and to develop clinical research programs for patients with sickle cell disease. The Clinic has detailed records for approximately 5000 patients, most of whom were ascertained as having sickle cell disease by symptomatic presentation, with long-term follow-up care over symptomatic and asymptomatic (steady-state) periods. The Jamaican Sickle Cell Cohort Study, previously described in detail, was initiated in 1973 and led to newborn screening in 100 000 consecutive deliveries. Approximately 550 subjects, with all genotypes of sickle cell disease, have been followed up since birth; the control population consists of 250 healthy persons with the AA phenotype.

Subjects

Adult patients with SS disease who were in steady state on the day of the clinic visit and who had no precluding medical condition were approached for study enrollment. Consecutive attendees during April 2000 (n = 83) and April to July 2002 (n = 274) provided 357 subjects who were pooled as the clinic sample because there were no apparent differences between the 2 groups. Data and DNA samples had been previously available for 209 SS patients from the Institute of Biological Anthropology and the Weatherall Institute of Molecular Medicine, University of Oxford, United Kingdom; the Tropical Medicine Research Institute, University of the West Indies, Mona, Kingston; and the Sickle Cell Trust, Kingston, Jamaica.


Supported by the Institute of Biological Anthropology, University of Oxford (R.W.), and by the Wellcome Trust and the Leverhulme Trust (D.J.W.). Supported in part by Medical Research Council, UK, grants G0001249 (T.F.) and G108/325 (C.A.M.).


Reprints: Eden V. Haverfield, Department of Human Genetics, University of Chicago, 920 E 58 St, CLSC 515, Chicago, IL 60637; e-mail: eden@genetics.uchicago.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology
subjects and 117 AA controls from the Jamaican Sickle Cell Cohort Study to create the cohort sample and the control sample, respectively. Participants gave written informed consent before blood collection. The Institutional Review Board of the University of the West Indies approved the study and the protocol for patient recruitment.

Clinical and laboratory data

Among the clinical variables extracted from the database were age, sex, and hepatobiliary complications, including gallstone status. Laboratory variables included hemoglobin level, reticulocyte count, fetal hemoglobin (HbF) level, and serum total and direct bilirubin levels. All hematologic indices were generated by standard methods, as previously described.21-23

For the Sickle Cell Clinic (clinic sample), hematology and bilirubin levels were determined every 1 to 2 years in the steady state, or more often if the subject was symptomatic. Only retrospective, steady-state bilirubin values were used for calculations of individual subject bilirubin means (average number of values per subject, 4.53 ± 5.50). Mean hemoglobin levels per subject were also calculated from retrospective, steady-state laboratory values. Gallbladder ultrasound examinations were performed only when clinically indicated. Clinical records for the clinic sample subjects were examined individually for reports of abdominal symptoms (right upper quadrant pain, increased jaundice, fever), gallstones, or other evidence of gallbladder disease.

For the Jamaican Sickle Cell Cohort Study, protocols required SS and AA subjects to be evaluated at 6-month intervals from the age of 5 years when they were clinically well and as necessary when symptomatic. Bilirubin values were tabulated from retrospective steady-state laboratory data based on an average of 15.74 ± 4.23 values for the cohort sample and 3.17 ± 1.76 values for the control sample. Mean hemoglobin levels were calculated as described. Gallbladder ultrasound examinations were performed annually by a single observer (T.W.) between 1987 and 2000.21

DNA extraction and genetic analysis

DNA was isolated from peripheral blood leukocytes of whole blood by standard phenol/chloroform methods.24 UGT1A1 (TA)n promoter length variability was genotyped in all samples by polymerase chain reaction (PCR)23 with forward primer 7F (5'-CAAGTGACGGACGATAC- CCGGGG) and the previously described reverse primer D (5'-GCGCCTTGCTCCTGCGCAGGGTT)4 to generate a 490-bp fragment and was subsequently sequenced. Each PCR reaction contained 40 ng genomic DNA template, 200 nM each primer (MWG, Ebersberg, Germany), 2.5 U HotstartTaq polymerase (ABGene, Epsom, United Kingdom), 200 μM dNTPs, 1× reaction buffer (ABGene), 1× glycerol, and ddH2O. PCR reactions were initially denatured for 10 minutes at 95°C; this was followed by 30 cycles at 95°C for 30 seconds, 64°C for 45 seconds, and 72°C for 1 minute and by a final 10-minute extension step at 72°C. The resultant fragment was purified using the QIAquick PCR purification kit (Qiagen, Crawley, United Kingdom) and was directly sequenced with the 7F-D primer pair using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, United Kingdom) and an ABI PRISM 3700 DNA analyzer (Applied Biosystems). Sequence analysis and alignments were performed with DNASTar software (DNASTar, Madison, WI) to detect the UGT1A1 promoter (TA)n length and any single nucleotide polymorphisms (SNPs) present in this region.

Statistical methods

Given that the bilirubin distributions were skewed, the Mann-Whitney U test was used to assess differences in bilirubin values among UGT1A1 (TA)n genotypes, with the homozygous (TA)n genotype considered the wild type. Among clinic subjects, we compared the proportions of symptomatic subjects and asymptomatic subjects with gallstones in each UGT1A1 (TA)n genotype with the proportions found in the homozygous (TA)n genotype. Among the cohort subjects, we compared the proportion of subjects with gallstones in each (TA)n genotype with the homozygous (TA)n genotype. In the clinic and the cohort samples, Fisher exact test was used to evaluate the significance of any differences observed. Odds ratios (with 95% confidence intervals [95% CIs]) were computed to describe the strength of the association between UGT1A1 (TA)n genotype and gallstone-positive ultrasound findings. To allow for multiple comparisons, only P less than .001 was considered significant at the 5% level.26 Conventional t tests were used to compare age, sex ratios, serum total and indirect bilirubin study group means, and hemoglobin levels. All statistical analyses were performed with SPSS (version 10.0; Woking, United Kingdom) or STATA (version 8.2; College Station, TX).

Results

Description of subjects

Clinical and laboratory variables were assessed for each sample group. Average subject age comparisons between the clinic sample (34.6 ± 10.9 years) and the age-matched cohort and control samples (25.0 ± 2.9 years and 25.4 ± 1.5 years, respectively) were significantly different (P = 2.20 × 10−21), a finding expected from the clinic sample cross-sectional recruiting scheme. Males were

<table>
<thead>
<tr>
<th>Allele</th>
<th>SS clinic sample</th>
<th>SS cohort sample</th>
<th>AA control sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>(TA)8</td>
<td>0.087</td>
<td>0.089</td>
<td>0.054</td>
</tr>
<tr>
<td>(TA)6</td>
<td>0.453</td>
<td>0.498</td>
<td>0.482</td>
</tr>
<tr>
<td>(TA)5</td>
<td>0.087</td>
<td>0.089</td>
<td>0.054</td>
</tr>
<tr>
<td>(TA)4</td>
<td>0.037</td>
<td>0.017</td>
<td>0.041</td>
</tr>
</tbody>
</table>

Table 1. UGT1A1 (TA)n allele frequency

Observed allele frequencies in 3 Jamaican groups determined by allele counting.

Figure 1. Bilirubin values. Box plots showing medians and interquartile ranges (IQRs, shaded regions) for (A) serum total and (B) indirect bilirubin values by study, with error bars indicating 1.5×IQR, shown on a log-scale on the y-axis. The horizontal line within the shaded regions is the median bilirubin level per genotype, with outliers denoted as filled circles. Numbers (N) with each genotype are indicated above each figure, with genotypes coded as 1/1; 2/2; 3/3; 4/4; 5/5; 6/6; 6/7; 7/6; 8/7; 9/7; and 10/8. *Genotypes are significantly different (P < .001) from genotype 5.
slightly more common in the cohort sample (52.6%) and the control sample (51.4%) than in the clinic sample (44.5%), but the difference did not reach a significant level. Mean serum total bilirubin levels were comparable in the SS groups (clinic sample, 3.08 ± 2.12 mg/dL; cohort sample, 2.81 ± 1.27 mg/dL) but were much higher than observed in the control sample (0.71 ± 0.53 mg/dL; P = 8.5 × 10⁻²¹). Similarly, indirect bilirubin levels were equivalent in the SS groups (clinic sample, 1.94 ± 1.54 mg/dL; cohort sample, 1.90 ± 1.20 mg/dL) and were lower in the control sample (0.40 ± 0.27 mg/dL; P = 2.1 × 10⁻²⁰). Mean bilirubin levels of the control sample were found to be in the reference range for serum total bilirubin (< 1.05 mg/dL) and indirect bilirubin (< 0.7 mg/dL) (Department of Chemical Pathology, University Hospital of the West Indies). Average hemoglobin levels were also significantly different in the control sample (11.4 ± 1.1 g/dL; P = 6.8 × 10⁻²¹) compared with the clinic and cohort samples (7.9 ± 1.2 g/dL and 8.0 ± 1.0 g/dL, respectively).

**UGT1A1 (TA)₆ promoter variation in SS and AA subjects**

We generated UGT1A1 (TA)₆ genotype data for 339 (95%) subjects in the clinic sample, 202 (97%) subjects in the cohort sample, and 111 (95%) subjects in the control sample. As previously identified in populations of African descent, ¹⁷⁻²⁰ (TA)₆-(TA)₆ alleles were present in these Jamaican samples, with (TA)₆ and (TA)₇ alleles predominant (Table 1). Observed genotype distributions of the clinic, cohort, and control samples were not significantly different from the values expected under Hardy-Weinberg equilibrium (data not shown). The 4 (TA)₆ alleles have been deposited in GenBank and assigned the accession number AY533178-81.

**UGT1A1 (TA)₆ promoter variation and bilirubin levels**

Homozygosity for the (TA)₇ allele and the presence of the (TA)₈ allele at the UGT1A1 locus were significantly associated with elevated levels of serum total and indirect bilirubin in the clinic sample and the cohort sample when compared with homozygous (TA)₆ subjects (Figure 1). The (TA)₆/(TA)₇ genotype had elevated levels of serum total and indirect bilirubin in the clinic sample but not in the cohort sample. This difference, which may reflect the older age and the larger number of subjects with this genotype in the clinic sample, or variation in ascertainment of the groups, is not significant after correction for multiple comparisons. Homozygosity for the (TA)₇ allele was also associated with increased serum total bilirubin levels in the control sample, but this effect was only of borderline significance (P = .004) after adjustment for multiple testing.

**UGT1A1 (TA)₆ promoter variation and gallstones in the SS population**

Evaluation of 339 records of the clinic sample revealed 89 subjects whose symptoms led to abdominal ultrasound examination; in 67 (75.3%) of these subjects, gallstones were detected at an average age of 29.2 ± 11.7 years. In this sample, homozygosity for the (TA)₇ allele was associated with an asymptomatic presentation in 50.7% compared with 85.0% in (TA)₆ homozygotes and 77.7% in those with the (TA)₆/(TA)₇ genotype (Table 2). Furthermore, 97.0% of the (TA)₇ homozygous symptomatic subjects were gallstone positive. In the cohort sample, gallstones occurred in 82 of 202 (40.6%) subjects, with an average age at diagnosis of 15.1 ± 4.7 years. Of the 82 gallstone-positive subjects, only 15 (18.3%) had gallstone-related symptoms. Homozygosity for the
(TA)7 allele was associated with a gallstone prevalence of 41.0% compared with 33.9% in (TA)6 homozygotes and 42.4% in those with the (TA)6/(TA)7 genotype (Table 3). These differences are not significant.

Point estimates for the odds ratios (Table 4) of having gallstones in subjects with the (TA)6/(TA)7 and (TA)7/(TA)7 genotypes compared with the (TA)6 wild type. Clinic sample (TA)7 homozygotes elevated in the (TA)7/(TA)8 genotype in the clinic (OR = 10.3; P = .003) samples, but they were not significant after correction for multiple testing. — indicates not calculated.

**Table 4. Odds ratios of gallstone risk in SS subjects**

<table>
<thead>
<tr>
<th>UGT1A1 genotype</th>
<th>SS clinic sample</th>
<th>95% CI</th>
<th>SS cohort sample</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/6</td>
<td>1.0</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>6/7</td>
<td>2.1</td>
<td>0.78-6.52</td>
<td>1.4</td>
<td>0.67-2.92</td>
</tr>
<tr>
<td>7/7</td>
<td>11.3</td>
<td>4.30-29.4*</td>
<td>1.4</td>
<td>0.64-3.49</td>
</tr>
<tr>
<td>7/8</td>
<td>6.7</td>
<td>1.84-24.6†</td>
<td>9.7</td>
<td>1.06-88.7†</td>
</tr>
</tbody>
</table>

Odds ratios and 95% CI for gallstone risk among subjects with symptoms (clinic sample) or for developing gallstones (cohort sample) by UGT1A1 (TA)7 genotype among clinic subjects with symptoms. Furthermore, the (TA)7/(TA)7 genotype was also significantly increased among the 75.3% symptomatic subjects who were found to have gallstones by abdominal ultrasound studies. While clinic subjects with symptoms were undoubtedly a biased sample and might have been expected to have a higher overall prevalence of gallstones, particularly because their mean age was 29.2 years, it is difficult to envision a mechanism whereby the UGT1A1 (TA)7/(TA)7 genotype would be overrepresented unless it was associated with survivorship (which seems unlikely given that the genotype frequencies in the clinic sample were not different from those in the cohort sample) or was causally related to gallstone formation and/or gallbladder disease.

These discrepancies raise some important issues regarding the diagnosis and natural history of gallstone formation in SS disease. In view of the association between UGT1A1 genotype and bilirubin levels, it is surprising that there was no association between gallstone frequency and UGT1A1 alleles in the younger cohort. Other factors may contribute to the evolution of gallstones, including a change in composition, size, or morphology of the gallstones, chronic infection of the gallbladder, or other genetic polymorphisms. These factors may interact over time with the high bilirubin levels associated with particular UGT1A1 alleles and may account for the high frequency of the (TA)7/(TA)7 genotype in older subjects with symptomatic gallstones.

Clearly, the role of increased bilirubin levels related to UGT1A1 promoter polymorphisms and gallstone formation is complex. Although these studies suggest that the (TA)7/(TA)7 genotype is related to symptomatic gallstone presentation in older subjects, further work is required to elucidate its role in gallstone formation and symptom manifestations in SS disease.

**Acknowledgments**

This work is dedicated to the memory of Professor Ryk Ward. We thank Dr Susanna Ali and the Sickle Cell Clinic Staff; Sharon Howell and Dr Kwesi Marshall for assistance in DNA extraction, and Ian Hambleton for assistance and helpful discussion. D.J.W thanks the Wellcome Trust and the Leverhulme Trust for support.

**References**


**Table 4. Odds ratios of gallstone risk in SS subjects**

<table>
<thead>
<tr>
<th>UGT1A1 genotype</th>
<th>SS clinic sample</th>
<th>95% CI</th>
<th>SS cohort sample</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/6</td>
<td>1.0</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>6/7</td>
<td>2.1</td>
<td>0.78-6.52</td>
<td>1.4</td>
<td>0.67-2.92</td>
</tr>
<tr>
<td>7/7</td>
<td>11.3</td>
<td>4.30-29.4*</td>
<td>1.4</td>
<td>0.64-3.49</td>
</tr>
<tr>
<td>7/8</td>
<td>6.7</td>
<td>1.84-24.6†</td>
<td>9.7</td>
<td>1.06-88.7†</td>
</tr>
</tbody>
</table>

Odds ratios and 95% CI for gallstone risk among subjects with symptoms (clinic sample) or for developing gallstones (cohort sample) by UGT1A1 (TA)7 genotype compared with the 6/6 wild type. P < .001 was regarded as significant after correction for multiple testing. — indicates not calculated.

1 *P = 7 × 10⁻⁴.
2 †P = .05 (clinical sample) or P = .03 (cohort sample).

**References**


**Table 4. Odds ratios of gallstone risk in SS subjects**

<table>
<thead>
<tr>
<th>UGT1A1 genotype</th>
<th>SS clinic sample</th>
<th>95% CI</th>
<th>SS cohort sample</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/6</td>
<td>1.0</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>6/7</td>
<td>2.1</td>
<td>0.78-6.52</td>
<td>1.4</td>
<td>0.67-2.92</td>
</tr>
<tr>
<td>7/7</td>
<td>11.3</td>
<td>4.30-29.4*</td>
<td>1.4</td>
<td>0.64-3.49</td>
</tr>
<tr>
<td>7/8</td>
<td>6.7</td>
<td>1.84-24.6†</td>
<td>9.7</td>
<td>1.06-88.7†</td>
</tr>
</tbody>
</table>

Odds ratios and 95% CI for gallstone risk among subjects with symptoms (clinic sample) or for developing gallstones (cohort sample) by UGT1A1 (TA)7 genotype compared with the 6/6 wild type. P < .001 was regarded as significant after correction for multiple testing. — indicates not calculated.

1 *P = 7 × 10⁻⁴.
2 †P = .05 (clinical sample) or P = .03 (cohort sample).

(Please note: The above text seems to be a mix of data and discussion, possibly due to incomplete or fragmented sentences. It might be a combination of a table and unstructured content, which requires interpretation and alignment to provide a coherent narrative.)


UGT1A1 variation and gallstone formation in sickle cell disease