Sickle cell disease (SCD) is a common inherited blood disorder in Saudi Arabia. SCD phenotype in Saudi Arabia is widely variable similar to other ethnicities. This indicates the involvement of other genes in determining SCD phenotype. Identification of genes that modify the phenotypic severity of monogenic diseases, SCD included, represents a paradigm shift in our view of these disorders and an important step toward a more meaningful phenotype/genotype correlation. Steady-state serum bilirubin level and risk of gallstones in SCD differ between patients. About 40% of SCD patients will develop gallstones, and the risk is associated partly with the intensity of hemolysis as shown by the protective effect of co-inheritance of α-thalassemia.

Polymorphism in the number of TA repeats (TA)n of UDP-glucuronosyltransferase 1A1 gene (UGT1A1) promoter was shown to modify the risk of gallstones and serum bilirubin level in SCD patients with African origin HBB haplotypes. The enzyme encoded by this gene glucuronidates unconjugated bilirubin to a water-soluble form. Mutation in the coding region of UGT1A1 causes Crigler-Najjar syndrome, and additional TA repeats in the UGT1A1 promoter is associated with Gilbert syndrome and higher serum bilirubin level in normal population.

There are no available data on the effect of the UGT1A1 promoter polymorphism on bilirubin level and risk of gallstones in patients with SCD in Saudi Arabia. Polymorphism in the number of TA repeats (TA)n of UGT1A1 promoter influences bilirubin level and risk of gallstones in patients with sickle cell disease (SCD) of African descent. Modifiers of bilirubin level and gallstones in Saudi patients with SCD are not known.
and probability of gallstones in Saudi SCD patients. In this report we describe the influence of the (TA)n UGT1A1 promoter polymorphism on serum bilirubin level and gallstones in 223 Saudi SCD patients from both the southwestern (SW) province with African origin HBB haplotypes and the eastern province with Arab-Indian (AI) haplotype.

METHODS

Patients

Patients with SCD (sickle cell anemia [HbSS], HbS-α0 thalassemia, and HbS-α+ thalassemia) presenting to participating institutions between July 2009 and July 2012 were enrolled in our study, after obtaining informed consent. The study was approved by our institutional review board. Saudi patients with both African origin HBB haplotypes (e.g., Benin, Bantu, and Senegal) and the Arab-Indian haplotypes were included in our study. The following information was obtained from all patients: age, gender, use of hydroxyurea, and presence of gallstones as confirmed by ultrasound during routine screening or in symptomatic patients.

Laboratory evaluation

Laboratory workup performed during steady state was collected (i.e., at least 3 weeks after acute events with no recent blood transfusion in the previous 3 months). DNA was extracted from peripheral blood of all patients using Gentra Puregene blood kit (Qiagen, Valencia, CA). Laboratory workup included complete blood count, reticulocytes, liver function tests, lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD) level, and hemoglobin (Hb) electrophoresis and was performed at participating institutions. Serum bilirubin was measured using the same method by diazo reaction using Dimension RxL analyzer (SIEMENS, Malvern, PA). Quality control is performed daily in our labs. HBB gene cluster haplotype was determined using restriction fragment length polymorphism technique as previously described,17 and the molecular diagnosis of common α-thalassemia deletions (αβ0, αβ+2, αβ+8, and αβ-MED) was performed by multiplex polymerase chain reaction assay.18 The presence of the non-deletional poly A signal mutation (αTS/αTS) was determined as previously described.19 All molecular studies were performed in our laboratory at King Saud University.

UGT1A1 promoter polymorphism

The UGT1A1 promoter region was amplified by polymerase chain reaction (PCR) in all patients to study the number of (TA)n in the UGT1A1 promoter using forward primer 5’- GAGGTTCGGAAGTACTTTTC -3’ and reverse primer 5’- CCAACATGCTCAGCCAG -3’ as previously described (12). PCR (20 µL) contained 1 µL of genomic DNA, 0.6 µL (10 µM) of each primer, 7.8 µL of H2O, and 10 µL of PCR mastermix (Norgen Biotek Corp. Ontario, Canada). Initial denaturation at 94°C for 5 minutes followed by 40 cycles (94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 60 seconds) then final 5 minutes at 72°C. PCR products were purified and subsequently sequenced using both primers. Sequencing was performed using 96 capillary gene analyzer (3730 Applied Biosystems, CA, USA). Data was analyzed using sequencing analysis software (v5.3.1) from Applied Biosystems, CA, USA).

Statistical analysis

The data analysis cutoff was July 10, 2012. Descriptive analyses involved the calculation of mean values (±1 standard deviation) and ranges for continuous data and frequencies for categorical data. The distribution of serum bilirubin was compared between groups using student t test for categorical predictors and simple linear regression for continuous predictors. The frequency of gallstones compared between groups via chi-squared testing for categorical predictors and simple logistic regression for continuous predictors. All variables with a P value ≤.05 on univariate analysis were included in the multivariate analysis. Multivariate analysis was performed using multiple regression for continuous variables and multiple logistic regression for categorical variables. Variables evaluated for influence on serum bilirubin level and risk of gallstones included age, gender, use of hydroxyurea, Hb level, white blood cells (WBC) count, platelet count, reticulocytes, fetal hemoglobin (Hbf), LDH, presence of G6PD deficiency, and co-inheritance of α-thalassemia. P value <.05 was considered statistically significant. Stata Statistical Software: Release 12 was used for all analyses (College Station, TX: StataCorp LP).

RESULTS

Patient characteristics

A total of 223 patients with SCD were enrolled in our study. Fifty-five percent of patients were females. The average age was 21.2 (12.4) years. Sixty-four percent of patients had HbSS, HbS-α0 thalassemia (26%), and HbS-α+ thalassemia (10%), based on Hb electrophoresis analysis in the absence of recent blood transfusion. The HBB gene cluster haplotype was Arab-Indian in (32%) and the remaining haplotypes were of African origin. Molecular testing for the presence of α-thalassemia
was performed in 124 patients; 38% of patients had co-inheritance of \( \alpha \)-thalassemia.

Sequencing of UGT1A1 promoter

TA6/6 repeat in the promoter region was the most common polymorphism and was found in 189 individuals (84.7%), TA7/7 in 26 (11.7%), TA5/5 in 6 (2.7%), and TA5/6 in 2 (0.9%). TA7/7 was relatively more frequent among patients with AI haplotype (18%) compared to SW individuals (9%). TA6/6 was found in 78% of AI patients compared to 88% in SW patients. TA5/5 was observed in 4% of AI patients compared to 1.9% in SW patients. The 2 patients with TA5/6 were from the SW region. TA8/8 repeat was not observed in our patient population.

UGT1A1 promoter polymorphism modifies bilirubin level

The steady-state bilirubin level was significantly higher among patients with TA7/7 (76 [39] \( \mu \)mol/L) compared to TA6/6 (32 [18] \( \mu \)mol/L) \((P<.0001)\) (Table 1 and Figure 1). The steady-state bilirubin level among 8 patients with TA5/5 or 5/6 was 22 (10) \( \mu \)mol/L. There were no differences in hemolytic markers (Hb, Reticulocytes, LDH), WBC, HbF, age, gender, and use of hydroxyurea between the 2 groups (Table 1). However, the steady-state platelet count was higher in patients with TA7/7 compared to TA6/6 \((P=.002)\). This difference in platelet count remained significant after adjusting for the presence of gallstones \((P=.04)\).

In addition to UGT1A1, the co-inheritance of \( \alpha \)-thalassemia was associated with lower bilirubin level \((P=.003)\), and the male gender had higher bilirubin level \((P=.02)\). There was positive linear relationship between bilirubin and LDH \((P=.001)\) and negative linear correlation with Hb level \((P=.009)\). Age \((P=.42)\), use of hydroxyurea \((P=.55)\), HbF \((P=.40)\), and presence of G6PD deficiency \((P=.67)\) did not influence bilirubin level. UGT1A1 \((TA)n \((P<.0001)\) and Hb level \((P=.005)\) remained significant on multivariate analysis. Of note, the UGT1A1 promoter polymorphism did not influence the degree of hemolysis, i.e., Hb and LDH levels.

UGT1A1 promoter polymorphism and risk of gallstones

Gallstones were more frequent in patients with TA7/7 (72%) compared to TA6/6; however, this difference did not reach statistical significance \((P=.22)\) (Table 1). Gallstones occurred in 37% of patients with TA5/5 or 5/6 and this was not significantly different than TA7/7 \((P=.18)\). Older age \((P=.0001)\) and absence of \( \alpha \)-thalassemia \((P=.03)\) were associated with a higher risk of gallstones. Other variables did not influence the risk of gallstones.

DISCUSSION

Serum bilirubin level and the risk of gallstones in SCD varies between patients similar to other SCD complications.\(^{1,20,21}\) Genetic modifiers that modulate serum bilirubin level and risk of gallstones in Saudi SCA are
not well defined. Factors that are associated with less intense hemolysis such as co-inheritance of α-thalassemia and higher steady-state Hb level were more frequent in patients without gallstones.\textsuperscript{1,2,22} In this study, we examined the impact of the number of TA repeats in the \textit{UGT1A1} promoter on the steady-state serum bilirubin level and gallstones.

TA6/6 was the most common polymorphism in Saudi patients similar to other population.\textsuperscript{8,10,11} TA8/8 was not observed in our population. Majority of our patients are homozygous for the same number of TA repeats, which might be related to high frequency of consanguineous marriages. The steady-state bilirubin level was significantly higher in patients with TA7/7 comparable to what have been observed in other ethnicities with SCD.\textsuperscript{4,6,9-11} Genome-wide association study (GWAS) in a large cohort of African Americans with sickle cell anemia showed significant association between single-nucleotide polymorphisms (SNPs) in \textit{UGT1A1} and serum bilirubin; however, these SNPs were not associated with hemolysis.\textsuperscript{5} In addition, male gender had higher bilirubin level, which is comparable to a previous report.\textsuperscript{11} The absence of α-thalassemia, higher LDH, and lower Hb, as markers of more intense hemolysis, were associated with higher bilirubin level among our patients.

TA7/7 or 8/8 in the \textit{UGT1A1} promoter was shown to increase the risk of gallstones in sickle cell anemia patients.\textsuperscript{4,6,9,10} In our study, there was a trend toward more frequent gallstones in patients with TA7/7 than TA6/6 or TA5/5. However, this did not reach statistical significance probably secondary to the small sample size, low frequency of TA7/7, and relatively young population (mean age 21 years). A Jamaican study reported association between TA7/7 and symptomatic gallstones in older patients and not in younger patients.\textsuperscript{6} The co-inheritance of α-thalassemia was associated with the lower risk of gallstones independent of (TA)n in the \textit{UGT1A1} promoter similar to a previous observation.\textsuperscript{8}

The impact of the high free unconjugated bilirubin observed with extra TA repeats in the \textit{UGT1A1} promoter on the clinical severity of SCD, other than gallstones, is not known. It is of interest that in our study, patients with TA7/7 had a higher platelet count compared to those with lesser number of repeats. This is independent of the presence of gallstones. So it is possible that the \textit{UGT1A1} promoter polymorphism is associated with inflammation leading to higher platelets. This is supported by a recent GWAS study that identified \textit{UGT1A1} as a modifier of the serum concentration of circulating cell-free DNA, which is a marker of cell death and can stimulate inflammation.\textsuperscript{23} This hypothesis, however, needs further investigation.

In conclusion, increased (TA)n in the \textit{UGT1A1} promoter and intensity of hemolysis are associated with higher serum bilirubin level, while the co-inheritance of α-thalassemia lessens the risk of gallstones in Saudi SCD patients.

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