

The Effect of *UGT1A1* Promoter Polymorphism in the Development of Hyperbilirubinemia and Cholelithiasis in Hemoglobinopathy Patients

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Abstract

Present study was aimed to explore the effect of (TA)_n *UGT1A1* gene promoter polymorphism on bilirubin metabolism, bilirubinaemia, predisposition to cholelithiasis and subsequent cholecystectomy, in Sickle-Cell Anemia (SCA) and beta-Thalassemia major (bTH) in Kuwaiti subjects compared to other population. This polymorphism was analyzed and correlated to total bilirubin and cholelithiasis in 270 age, gender, ethnically matched subjects (92 bTH, 116 SCA and 62 Controls) using PCR, dHPLC, fragment analysis and direct sequencing. Four genotypes of *UGT1A1* were detected in this study (TA6/6, TA6/7, TA6/8 and TA7/7). (TA)6/8 was found only in four individuals; hence it was not included in the analysis. There was a statistically significant association of genotypes with serum total bilirubin levels in both bTH and SCA groups ($p < 0.001$). Subjects with (TA)7/7 had the highest total serum bilirubin level ($178.7 \pm 3.5 \mu\text{mole/l}$). A significant association was observed between allele (TA)7 and cholelithiasis development ($p = 0.0001$). The 40%, 67.5% and 100% of SCA with (TA)6/6, (TA)6/7 and (TA)7/7 respectively developed cholelithiasis and were subsequently cholecystectomized. Our results confirm *UGT1A1* (TA)7 allele as one of the factors accounting for the hyperbilirubinemia and cholelithiasis observed in SCA and bTH.

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Introduction

Bilirubin is a toxic metabolite, predominantly resulting from the turnover of hemoglobin. Uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*), the A1 isoform encoded by the bilirubin UDP glucuronosyltransferase 1 family (*UGT1*), catalyzes glucuronidation of bilirubin in humans through the formation of intermediate derivatives mono- and diglucuronides [1]. The Elevation of unconjugated bilirubin due to inadequate bilirubin glucuronidation is associated with the accumulation of serum bilirubin, [2] this unconjugated hyperbilirubinemia results from decreased activity of *UGT1A1* enzyme to approximately 30% of normal levels [3].

UGT1A1 is encoded by *UGT1A1* gene, which consists of five exons and is a part of the *UGT1A* locus on chromosome 2q37 [4,5]. Polymorphisms in *UGT1A1* gene promoter have been shown to affect transcriptional efficiency, strongly influencing bilirubin metabolism and clearance [6]. The wild type promoter contains an A (TA) nTAA sequence with (TA)6 repeats, while a less frequent allele contains extended repeat sequence (TA)7. The homozygous genotype of the latter allele was associated with unusually high levels of bilirubin and a significantly increased frequency of gallstones and gall bladder disease [6–8]. The (TA)7 allele has also been associated with increased bilirubin levels in apparently healthy individuals. Two other alleles, (TA)5 and (TA)8, also been identified, primarily in individuals of African descent [9].

Beta Thalassemia major is a quantitative problem with a genetic defect that results in reduced rate of synthesis of the two beta globin chains causing severe anemia, often through mutations in regulatory genes. On the other hand, sickle-cell anemia (a hemoglobinopathy) is a qualitative problem of synthesis of an incorrectly functioning globin with mutation of the sixth amino acid valine to glutamine. The High levels of erythrocyte destruction in patients with SCA and to a lesser extend in bTH result in chronic hyperbilirubinemia. A significant proportion of patients are prone to cholelithiasis due to high biliary concentration of unconjugated bilirubin, which tends to coprecipitate with calcium in the gall bladder lumen. Cholelithiasis, by promoting cholecystitis and choledocholithiasis, is responsible for high levels of morbidity in hemoglobinopathy patients [2] and elective cholecystectomy is therefore recommended for patients developing this complication [10]. The coinheritance of aforementioned hematological diseases and *UGT1A1* gene promoter A (TA) 7TAA polymorphism will probably increase the risk of developing cholelithiasis in such patients [2,6–8].

No data have so far been reported about the prevalence of *UGT1A1* (TA)_n polymorphism in the Kuwaiti population except for one article screened the Kuwaiti G6PD patients for *UGT1A1* (TA)_n polymorphism without including healthy control [11]. Herein we carried out a population study to screen the frequency of different (TA) repeats in Kuwaiti population compared to other ethnicities. We have further analyzed the correlation between this

polymorphism and hyperbilirubinemia and the prevalence of cholelithiasis in the tested SCA and bTH patients.

Results

Screening region of interest revealed the existence of four genotypes of *UGT1A1* (TA)_n polymorphism in Kuwaiti population (6/6, 6/7, 6/8 and 7/7). Genotype (TA) 6/8 was detected only in 4 out of 236 tested cases; hence it was excluded from the analysis. Genotype (TA) 6/7 was predominant in both tested cases (≥63%) and controls (55%) while (TA) 7/7 was found to be rare (<6%) in both cohorts (table 1). Observed allele and genotype frequencies of *UGT1A1* (TA)_n polymorphism failed to show any significant difference between the tested subjects (*p*>0.05) with respect to age or gender.

To evaluate the association of *UGT1A1* (TA)_n polymorphism with hyperbilirubinemia, serum total bilirubin of each tested subjects were detected. A significant association of average serum total bilirubin was observed with bTH and SCA subjects (*p*<0.05). Average serum total bilirubin was higher in SCA (57.12±39 μmol/L, *p*<0.0001) than in bTH (28.87±20.4 μmol/L, *p*=0.001), when compared individually to healthy control (14.34±2.20 μmol/L) (figure 1). As shown in table 1 subjects with genotype (TA)7/7 had the highest levels, while (TA)6/7 had intermediate levels and the lowest level was found in genotype (TA)6/6. The average serum total bilirubin was significantly higher in bTH with 6/6 (*p*=0.0014) and 6/7 genotype (*p*=0.0001) and SCA with 6/6, 6/7 and 7/7 genotype (*p*=0.0001) when compared to individually to the healthy control with respective genotype. There was a significant association between the various genotypes of *UGT1A1* (TA)_n polymorphism and the serum total bilirubin levels in both bTH and SCA subjects (*p*<0.001), however no significant difference was observed based on gender.

All recruited SCA cases (104) underwent liver/biliary ultrasound scans and their data was available. A 67.3% of 104 of the tested SCA patients had gallstone disease. SCA patient having cholelithiasis had significantly higher serum total bilirubin (66.5±44.7 μmol/L), conjugated bilirubin (16.3±17.6 μmol/L) and unconjugated bilirubin concentrations (50.7±29.6 μmol/L) than the patients without cholelithiasis (Table 2). No significant difference in hemoglobin concentration, RBC or WBC count was

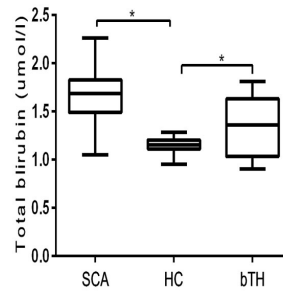


Figure 1. Represents serum total bilirubin in SCA, HC and bTH. A significant association of serum total bilirubin was observed with bTH (*p*=0.001) and SCA subjects (*p*<0.0001) when compared individually to healthy control by ANOVA test. *Indicates Significance *p*<0.05. doi:10.1371/journal.pone.0077681.g001

observed between the tested subjects. When student’s t-test was computed, the level of total serum bilirubin and its two forms were significantly higher in patient with cholelithiasis with *p*<0.05 (Table 2). The frequency of cholelithiasis in the study groups revealed; 40% SCA patients with *UGT1A1* (TA)6\6 genotype developed gallstones, 76.5% heterozygotes (TA)6\7 and 100% homozygous SCA patients with *UGT1A1* (TA)7\7 had cholelithiasis (Table 3).

Table 2. Bilirubin levels (μmol/L) in SCA patients with and without gallstone disease.

Serum Bilirubin	Gall Stone disease		<i>p</i> -value*
	Present (n = 70)	Absent (n = 34)	
Total Bilirubin	66.5±44.7	37.1±13.7	0.011
conjugated Bilirubin	16.3±17.6	5.4±.94	0.014
unconjugated Bilirubin	50.7±29.6	31.7±13.3	0.015

*Students t-test. doi:10.1371/journal.pone.0077681.t002

Table 1. Allele and genotype frequencies of *UGT1A1* promoter polymorphism in β-Thalassemia, Sickle Cell Anemia and Healthy Controls (# total Bilirubin measured in μmol/L).

Genotype	bTH (n = 70)		SCA (n = 104)		HC (n = 62)	
	Freq n (%)	Total Bilirubin	Freq n (%)	Total Bilirubin	Freq n (%)	Total Bilirubin
6/6	18 (27)	20±9	30 (29)	30.1±13.1	26 (42)	13.9±1.38
6/7	44 (63)	28.5±17.7	68 (65)	58±26.4	34 (55)	15.2±2
6/8	4 (5)	28.5±15	0	–	0	–
7/7	4 (5)	71±43	6 (6)	178.7±3.5	2 (3)	15
Allelotype	Freq n (%)		Freq n (%)		Freq n (%)	
6	84 (59)		124 (60)		86 (67)	
7	52 (38)		84 (40)		38 (33)	
8	4 (3)					

n= Number of samples of a particular genotype/allelotype. Freq = Frequency. doi:10.1371/journal.pone.0077681.t001

Table 3. Allele and genotype frequencies of *UGT1A1* promoter polymorphism in SCA diagnosed with or without gall stone.

Genotype	Cholelithiasis	Cholelithiasis	<i>p</i> -value	OR	95% CI
	Positive (n = 70)	Negative (n = 34)			
6/6	12 (40%)	18	0.0001	0.184	0.07–0.46
6/7	52 (76.5%)	16	0.006	3.25	1.37–7.69
7/7	6 (100%)	0	–		
Allele					
6	76	52	0.002	0.36	0.19–0.70
7	64	16		2.74	1.42–5.25

Dominant model 6/6 versus (6/7+7/7) showed increased susceptibility to cholelithiasis ($p = 0.0001$, $OR = 5.44$, $95\% CI 2.17–13.59$).

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A significant association was observed between having allele (TA) 7 and the development of gallstone in SCA patients ($p = 0.002$, $OR = 2.74$, $95\% CI 1.42–5.25$) table 3. The development of symptomatic bilirubin gallstones requiring cholecystectomy was significantly higher for patient with the (TA) 7/7 genotype than those with the (TA) 6/6 or (TA) 6/7 genotype ($p < 0.05$). An increased risk for cholelithiasis was observed among SCA when dominant genetic model was employed (6/6 versus (6/7+7/7) $p = 0.0001$, $OR = 5.44$, $95\% CI 2.17–13.59$), indicating the risk role of allele 7 in the development of gall stone.

Association of *UGT1A1* promoter polymorphism and serum total bilirubin with tested subjects was further assessed using logistics regression by adjusting for factors such as age and gender (Table 4). Analysis of *UGT1A1* polymorphism revealed no significant association with the studied diseases. However, average total serum bilirubin showed significant association with both bTH ($p = 0.016$, $OR = 1.036$) and SCA ($p < 0.0001$, $OR = 1.80$).

Our results in general sheds light on the fact that longer *UGT1A1* repeat, increases the risk of developing hyperbilirubinemia and cholelithiasis irrespective of disease status. The coinheritance of *UGT1A1* polymorphism (7/7 or 6/7 repeat) and SCA was found to potentially increase the risk of developing cholelithiasis ($OR = 5.44$). We further assessed the global frequency of the genotype distribution of *UGT1A1* promoter polymorphism according to the location on the various continents (Table 5) [9,11–35]. When comparing the Kuwaiti population with the Yemenis ($p = 0.448$), the origin of all Arabs and other Arabic population revealed no significant difference. Similarly, no significant difference was found between the Kuwaitis and the Caucasian populations ($p > 0.05$). A highly significant differences was found between Kuwaitis, Asians, Africans, southern Americans and the pacific islands (Papua New Guinea Tonga Fiji) populations $p < 0.05$ [9].

Discussion

The theory of single gene disorders is no more accurate as the mutation in the globin genes alone is not sufficient to account for one of the major characteristics of RBC disorders such as bTH and SCA manifestations. There is a wide range of phenotypic expression of the disease, even in patients with identical hemoglobin genotypes from apparently similar environments [36]. This strongly suggests that modifier genes other than beta globin gene play a role in the phenotypic diversity within bTH,

Table 4. Analysis of *UGT1A1* promoter polymorphism in SCA and bTH subjects using logistic regression.

SCA	<i>p</i> -value	AOR*	95% CI
Age	0.98	0.998	0.87–1.14
Gender			
Male	Reference		
Female	0.331	2.069	0.48–8.96
Total bilirubin	<0.0001	1.800	1.39–2.34
Genotype			
6/6	Reference		
6/7	0.106	3.481	0.77–15.82
7/7	0.965	1.146	0.003–510.6
bTH	<i>p</i> -value	AOR*	95% CI
Age	0.343	0.958	0.88–1.05
Gender			
Male	Reference		
Female	0.953	1.024	0.47–2.24
Total bilirubin	0.016	1.036	1.01–1.07
Genotype			
6/6	Reference		
6/7	0.199	1.676	0.76–3.69
7/7	0.363	2.362	0.37–15.08

*Odds ratio adjusted by age and gender.

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SCA as well as other hemoglobinopathies. We have recruited patients with bTH and SCA, followed their clinical data and hematologic/biochemical parameters for almost one to five year. The medical follow-up of SCA patients included the hepatobiliary ultrasound scan to assess the cholelithiasis.

Several risk factors for cholelithiasis have been identified in previous studies, one of which is the high total [37,38] and unconjugated bilirubin concentrations [38,39]. In our study, cholelithiasis was associated with high total bilirubin concentrations in SCA. No variation was noticed in terms of age and gender. We further evaluated the contribution of *UGT1A1* genotype to unconjugated hyperbilirubinemia and to the prevalence of cholelithiasis. Three (TA6, TA7 and TA8) of the four known alleles in the *UGT1A1* promoter region and four (TA6/6, TA6/7, TA6/8 and TA7/7) of the six possible genotypes were retrieved. We compared patients with and without cholelithiasis and found that the frequency of *UGT1A1* alleles (TA)6 was lower, and that of alleles (TA)7 was significantly higher in patients with cholelithiasis ($p = 0.002$, $OR = 2.74$, $95\% CI 1.42–5.25$). These data suggest a possible relationship between the *UGT1A1* locus and the occurrence of cholelithiasis in patients with SCA and bTH and most probably other hemoglobinopathies. Few studies have shown that allele (TA)7 is associated with high steady state concentrations of unconjugated bilirubin in serum and is a risk factor for cholelithiasis in adults with SCA or bTH. Furthermore, the importance of the pharmacogenomic effect of *UGT1A1* polymorphism was shown in modulating the hematologic response to hydroxyurea treatment in SCA patients [40]. Our study included patients with bTH and SCA and confirmed genetic risk factors for cholelithiasis in SCA, provided information about all the *UGT1A1* genotypes identified and strongly suggested that *UGT1A1*

Table 5. Global distribution of the *UGT1A1* promoter TATA box polymorphism.

Continent	Country	Total	6/6(%)	6/7(%)	7/7(%)	4/4(%)	5/5(%)	5/6(%)	5/7(%)	5/8(%)	6/8(%)	7/8(%)	8/8(%)	Ref	
Europe	Iceland	69	29 (42)	33 (47.8)	7 (10.1)									9	
	Uk	59	30 (50.8)	26 (44)	3 (5)									9	
	Uk2	81	10 (12.3)	26 (32)	18 (22.2)		4 (5)	13 (16.2)	4 (5)		1 (1.2)	5 (6)		12	
	Greek cypriots	47	23 (48.9)	19 (40.4)	4 (8.5)								1 (2.1)	9	
	Greek 2	152	74 (48.7)	51 (33.5)	27 (17.8)									13	
	Greek 3	37	18 (48.6)	12 (32.4)	7 (18.9)									14	
	Basque	27	13 (48)	10 (37)	4 (14.8)									9	
	Catalan	46	14 (30)	29 (63)	3 (6.5)									9	
	Austria	255	94 (37)	127 (50)	33 (13)									15	
	Croatia	1109	210 (19)	292 (26.3)	600 (54)				4 (0.36)		1 (0.09)	2 (0.18)		16	
	Italian	98	43 (43.9)	39 (39.8)	16 (16.3)									17	
	Slovenian	236	90 (38.1)	113 (47.9)	32 (13.6)									18	
	Southern Germany	265	112 (42.3)	121 (45.7)	32 (12)									19	
	Caucasian Germany	Caucasian Germany	100	50 (50)	42 (42)	8 (8)									20
		Dutch Caucasian	430	190(44.2)	188(43.7)	51(11.9)									21
Netherlands		41	17 (41)	18 (44)	6 (15)									22	
Africa and African Origin		Kenya (Luo)	81	17 (19.1)	32 (35.9)	16 (17.9)		1 (1.12)	5 (5.6)	7 (7.8)	2 (2.2)				9
		Malawi	76	14 (18.9)	35 (47.2)	7 (9.4)			8 (10.8)	5 (6.7)	1 (1.3)	4 (5.4)	2 (2.7)		9
	Ivory coast	74	17 (22.9)	26 (35.1)	6 (8.1)			3 (4)	5 (6.7)	1 (1.3)	6 (8.1)	10 (13.5)		9	
	Jamaica	72	22 (30.5)	22 (30.5)	7 (9.7)		2 (2.7)	8 (11.1)	5 (6.9)	1 (1.3)	2 (2.7)	2 (2.7)	1 (1.3)	9	
	Madagascar	67	40 (59.7)	19 (28)	4 (5.9)			3 (4.4)					1 (1.4)	9	
	Nigeria	226	28 (12.6)	88 (39.6)	45 (20.3)		2 (0.9)	26 (11.7)	15 (6.8)	1 (0.5)	5 (2.3)	11 (5)	1 (0.5)	23	
	Asia	Hong Kong	50	38 (76)	11 (22)	1 (2)								9	
China Shanghai	1035	838 (81)	172 (16.6)	25 (2.4)									24		
Japan	36	35(97.2)	1 (2.8)										25		
Thailand	76	60 (78.9)	14 (18.4)	2 (2.6)									9		
Indonesia	60	40 (66.6)	17 (28.3)	3 (5)									9		
Korea	20	17 (85)	2 (10)	1 (5)									26		
Vietnam	83	70 (84.3)	12 (14.4)	1 (1.2)									9		
India 1	119	45 (37.8)	51 (42.8)	23 (19.3)									9		
India 2	50	25 (50)	21 (42)	4 (8)									27		
India 3	95	32 (33.6)	53 (55.7)	10 (10.5)									28		
Srilanka	229	58 (25.3)	116 (50.6)	55 (24)									9		
Bangladesh	26	6 (23)	15 (57.6)	5 (19.2)									9		
Myanmar	32	22 (68.75)	9 (28.12)	1 (3.1)									9		
Turkey	32	18 (56)	11 (34)	3 (10)									29		
Lebanon	42	16 (38)	22 (52.3)	4 (9.5)									9		
Egyptian	50	56 (64.8)	36 (38.4)	8 (6.8)									19		
Yemen	61	33 (54)	25 (40.9)	3 (4.9)									9		
Kuwait	62	26 (42)	34 (55)	2 (3)									*		
South America	Amerindians	59	33 (55.9)	18 (30.5)	7 (11.8)							1 (1.6)		9	
	Brazil													30	
	Caucasians	71	32 (45)	28 (39)	9 (12.6)			1 (1.4)			1 (1.4)			30	
	African derived	54	13 (24)	25 (46)	9 (16)			5 (10)	1 (2)	1 (2)				30	
	Parakana Indians	32	12 (38)	19 (59)	1 (3)									30	

Table 5. Cont.

Continent	Country	Total	6/6(%)	6/7(%)	7/7(%)	4/4(%)	5/5(%)	5/6(%)	5/7(%)	5/8(%)	6/8(%)	7/8(%)	8/8(%)	Ref
North America	North Carolina	101	37 (36.6)	39 (38.6)	13 (12.9)		1 (0.9)	3 (2.9)	1 (0.9)		3 (2.9)	4 (3.9)		31
	North Carolina	2200	56 (28)	72 (36)	33 (16.5)		1 (0.5)	19 (9.5)	10 (5)		4 (2)	5 (2.5)		32
	Mexico	375	155 (41.3)	174 (46.4)	38 (10.1)			7 (1.9)	1 (0.3)					33
	Boston (African American)	609	276 (45.3)	272 (44.7)	60 (9.8)									34
	New York	32	9 (28.25)	11 (34.25)	2 (6.25)		2 (6.25)	4 (12.5)	2 (6.25)			2 (6.25)		35
Pacific	Papua New Guinea	105	102 (97.1)	3 (2.8)	0 (0)									9
	Tonga	41	32 (78)	8 (19.5)	1 (2.4)									9
	Fiji	16	15 (93.7)	1 (6.25)	0 (0)									9
Chimpanzees		35					35 (100)							9

*current study

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promoter polymorphism is a significant non-globin genetic modifier in these cases.

Two global surveys showed a wide variation in the frequency of the number of (TA) repeats in the promoter region of the *UGT1A1* gene [9,41]. We have further aimed to update the survey and compared the data obtained from Kuwaiti population with other ethnicities (table 5). (TA)6 is the most common allele in all studied populations whereas (TA)7 is the highest in the Croatian population where more than half (54.1%) of this population have homozygous (TA)7/7. The Srilankian (24%), Bangladesh (19.2%) and a subpopulation of the India (19.3%) also have a high frequency of homozygous (TA)7/7. A subset of the UK population (22.2%) and the Nigerian population (20.3) has a high frequency of homozygous (TA)7/7. Allele (TA)7 was rarest in Pacific islands (0%) (Papua New Guinea and Fiji) and also in Asians particularly Vietnamese and Chinese populations while has a highly variable frequency in European populations. In African populations, the spectrum is quite broad, ranging from five to eight (TA) repeats. The Kuwaiti population was the closest to other Arabic populations and to the Caucasians. A highly significant differences was found between Kuwaitis and Asians, Africans (Nigerian, Merganser, Kenya), southern Americans and the pacific islands (Papua New Guinea Tonga, Fiji) populations $p < 0.05$. However, Africans Malawai and the Jamaicans have not shown significant difference with the Arab and Caucasian populations.

Table 6. Characteristics of study subjects.

Factors	bTH (n=70)	SCA (n=104)	HC (n=62)
Age	29.37±2.47	31.24±4.93	30.58±5.75
Gender (male)	37% (26)	47% (48)	35.5% (22)
Ethnicity	Arabs		
WBC ($\times 10^9/l$)	7.89±3.04	8.79±3.45	8.56±1.79
RBC ($\times 10^{12}/l$)	4.10±0.81	3.99±0.98	4.23±0.308
Hb level(g/l)	124±5.62	126±18.9	128±18.9
Gall stone	–	67.3% (70)	–

bTH- b-thalassemia, SCA- Sickle cell Anaemia, HC- Healthy control.

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Materials and Methods

A total of 270 subjects were recruited in the study (116 SCA, 92 homozygous bTH and 62 health controls) after obtaining informed consent. Of these, 236 subjects who fit the selection criteria were carefully selected. All subjects were adult (>26 years of age) Kuwaiti Arabs with 1:1.5 male to female ratios. The patients were regularly transfused with packed red cells every 4 weeks to maintain mean hemoglobin levels above 120 g/l, and were receiving regular iron chelating therapy (deferoxamine mesylate 40 mg/kg daily). Blood was withdrawn from the patients before receiving transfusion. Healthy Subjects were excluded if they had a past or present history of hepatic/hematological disease. None of the recruited bTH and SCA subjects had a history of primary hepatic disorders, excessive alcoholism, chronic use of medications or narcotics, nor had received any drug two weeks prior to our investigation. Clinical characteristics of each subject selected for the study is detailed in table 6. Fifty nine percentage of the recruited hemoglobinopathy cases underwent liver/biliary ultrasound scans (104/174) to assess the cholelithiasis. A 67.3% of 104 SCA patients had gallstone disease. Written informed consent was obtained from each participant, under the protocols approved by the Joint Committee for the Protection of Human Subjects in Research.

Measurement of Serum Bilirubin Levels

After an overnight fast, Blood and serum samples were collected from each subject in EDTA treated and plain tubes respectively. Plasma concentrations of total bilirubin were determined by diazo method at least three times within 6 months. Mean values were considered for the comparative study. Liver function tests were performed in the hospital laboratory by a standardized colorimetric procedure LXI, Beckman biochemical analyzer.

Genotyping of UGT1A1 (TA)_n Promoter Polymorphism

DNA was extracted from the blood samples using the QIAamp Mini DNA extraction kit (QIAGEN, Germany). The forward primer 5'-GAGGTTCTGGAAGTACTTTTGC-3' and the reverse primer 5'-CCAAGCATGCTCAGCCAG-3' were used to amplify the region of interest in the 5' region of the UGT1A1 promoter fragment of 409 bp. The PCR amplicons were subjected to dHPLC (denaturing high-performance liquid chromatography) to screen for the number of repeats in the TATA box. The

presence of two well-resolved peaks reveals the heterozygous condition such as (TA)₆/(TA)₇. The presence of one peak is characteristic of the homozygous condition. To distinguish (TA)₆ from (TA)₇ homozygotes, each sample showing a single peak was mixed with (TA)₆/(TA)₆ control DNA under conditions allowing heteroduplex formation. The homozygous condition for (TA)₇ was revealed by a double peak, whereas for (TA)₆ homozygotes, there was no change in the chromatogram showing a single peak. Another screening method we used was the fragment analysis where the forward primer used was labeled with FAM fluorescent dye and amplicons were subjected to capillary electrophoresis. Genotyping results for 60 randomly selected samples (25% of tested cases) were further confirmed by sequencing using genetic analyzer ABI 3100 (Applied BioSystem, USA). The dHPLC results showed a 100% match with those obtained by direct sequencing and fragment analysis.

Statistical Analysis

Data collection, data management and statistical analysis were performed with SPSS 19.0 (SPSS, Inc., Chicago, IL). Allele and genotype frequencies of *UGT1A1* promoter polymorphism were calculated manually for each subject. Observed genotypes showed no significant deviation ($p > 0.05$) from Hardy Weinberg equilibrium by Genepop software. Differences in the genotypic and the allelic frequency between the patients and controls were assessed by the chi-squared p value test. A p -value of < 0.05 was considered to be statistically significant. Average serum total bilirubin is expressed as mean values \pm Standard deviation (unless stated

otherwise). Test for homogeneity ($p < 0.05$) indicated asymmetric distribution, hence the total bilirubin data was log transformed and the statistical difference between the compared subgroups were assessed either by one-way ANOVA test or students t -test. Association of genotypes and total serum bilirubin level to disease status were assessed by binary logistic regression adjusting for factors such as gender and age.

Conclusion

The frequency of *UGT1A1* (TA) _{n} promoter polymorphism genotypes was determined for the first time in healthy Kuwaiti population, and is similar to frequencies observed in Caucasian populations. The extremely rare (TA)₈ allele in Caucasians were also found in Kuwaitis. We further reveal that bTH and SCA patients (regardless of the age or gender) can be classified into three risk groups according to *UGT1A1* genotype. Patients homozygous for (TA)₇ are associated with a high frequency of cholelithiasis. *UGT1A1* genotyping is therefore a potentially useful tool for identifying individuals with hemoglobinopathy at high risk of cholelithiasis and requiring close clinical monitoring.

Author Contributions

Conceived and designed the experiments: SA. Performed the experiments: SA HA MH MM RN. Analyzed the data: SA HA MH. Contributed reagents/materials/analysis tools: SA HA MM. Wrote the paper: SA RN MM.

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