Gilbert syndrome acts as a risk factor of developing gallstone among β hemoglobinopathy Tunisian patients.

Le syndrome de Gilbert est un facteur de risque pour la formation de lithiases biliaires chez des patients Tunisiens atteints d'une β hemoglobinopathie

Chaouch Leila, Kalai Miniar, Chaouachi Dorra, Mallouli Fethi, Hafsia Raouf, Ben Ammar Slim, Abbes Salem.

Laboratoire d'hématologie moléculaire et cellulaire.
Institut Pasteur Tunis

RÉSUMÉ

Prérequis : L'hémolyse chronique entraine une hyperbilirubinémie qui causerait la formation de lithiases biliaires. Un déficit au niveau de l'enzyme uridine diphosphoglucuronosyltransférase 1A1 est un facteur aggravant pour la survenue de cette complication.

But : Dans ce travail, nous avons étudié l'effet de la mutation de gilbert sur la survenue de lithiases biliaires chez des patients atteint d'une hémoglobinopathie incluant la β thalassémie mineure et la drépanocytose.

Matériel et méthodes : L'étude a concerné 151 patients composés de 75 drépanocytaires et 76 β thalassémiques mineure. Les deux groupes de patients sont divisés en deux sous groupes selon la présence ou l'absence de lithiases biliaires. On a cherché une corrélation entre la variation A(TA)nTAA du gène UGT1A1 et la formation de la lithiase biliaire.

Résultats : Nos résultats ont montré une association significative entre les génotypes ayant le variant de gilbert (TA)7 et l'hyperbilirubinémie. Nous avons montré aussi une association entre les génotypes (TA)6/(TA)7 et (TA)7/(TA)7 avec la survenue de la lithiase biliaire chez les drépanocytaires et les β thalassémiques mineures ($p<0.05$).

Conclusion: Nos résultats confirment l'implication de la mutation de gilbert dans la formation des lithiases biliaires chez les patients drépanocytaires et β thalassémiques mineures.

Mots-clés

Drépanocytose, β Thalassémie mineure, Syndrome de Gilbert, Lithiases Biliaires, glucuronidation de la bilirubine, UGT1A1.

SUMMARY

Background: As a result of chronic hemolysis, hyperbilirubinemia is often observed, leading to the formation of pigment cholelithiasis which could be busted by the presence of uridine diphosphoglucuronosyltransferase 1A1 defects.

Aim: Herein, we investigated the effect of glibert mutation on the occurrence of pigment cholelithiasis in Tunisian patients with beta (β) hemoglobinopathy including sickle cell anemia and β thalassemia (minor).

Subjects and methods: Our study included 151 subjects divided in 75 SCA patients and 76 β thalassemia patients. Both groups of patients were divided into two sub-groups according to the presence or absence of cholelithiasis. The relationship between A(TA)nTAA variation of UGT1A1 gene, the serum bilirubin level and the occurrence of cholilithiasis was investigated.

Results: Our results show a significant association between genotypes carrying variant (TA)7 and hyperbilirubinemia ($p<0.05$). Furthermore, we demonstrated a significant association between (TA)6/(TA)7 and (TA)7/(TA)7 genotypes with cholelithiasis among sickle cell anemia and thalassemia patients ($p<0.05$).

Conclusion: Altogether, our data provide evidence that genotypes (TA)6/(TA)7 and (TA)7/(TA)7 and (TA)7 variant present a risk factor of developing gallstone among β hemoglobinopathy Tunisian patients.

Key-words

Sickle cell anemia, βthalassemia, cholelithiasis, Gilbert Syndrome, Bilirubin glucuronidation, genetic variation, UGT1A1.

Sickle cell anemia (SCA) and thalassemia are the most common hemoglobinopathies in the world [1]. Haemoglobin disorder is a chronic hemolytic disease. As a result of chronic hemolysis, hyperbilirubinemia is often observed leading to the formation of pigment cholelithiasis, which could be busted by the presence of uridine diphosphoglucuronosyltransferase 1A1 (UGT1A1) defects. Accordingly, UGT1A1 gene encodes the UDP-glucuronosyltransferase, enzyme responsible of bilirubin glucuronidation (B-UGT) (EC 2.4.1.17) [2]. UGT1A1 gene is located in 2q37 [3].Various UGT1A1 gene defects and polymorphisms have been described leading to a reduced enzyme activity [4]. Among these, a variation in the number of TA repeat at the A (TA)6TAA normal nucleotide sequence in the promoter region has been depicted. Interestingly, the addition of an extra (TA) at this sequence, called variant A(TA)7TAA or (TA)7, has been described to cause reduced glucuronidation and hyperbilirubinemia associated with Gilbert syndrome [5,6]. This variation at the promoter seems to interfere with binding of the transcription factor IID and results in reduced expression of bilirubin-UGT1 (30% of normal) [5]. Several studies have confirmed the implication of variant (TA)7 in the occurrence of gallstones out of hemolytic disorders [7-10]. Besides, other studies have shown the correlation of cholelithiasis and A(TA)7TAA variant of UGT1A1 promoter with chronic hemolytic disease such as thalassemia minor, which represent a risk factor for cholelithiasis and the Gilbert mutation further increases this risk By cons, no studies have been published to date about the correlation between (TA)7 and gallstones formation among β hemoglobinopathy Tunisian patients.

Herein, we aimed to study the impact of A(TA)nTAA variation at the UGT1A1 gene promoter in the occurrence of cholelithiasis among sickle cell and β thalassemia (minor) patients in Tunisia.

SUBJECTS AND METHODS

Subjects

The study enrolled 151 β hemoglobinopathy patients including 75 SCA patients (SS) and 76 β thalassemia (minor). SCA patients were selected on the basis of homozygosity for βs-globin gene (codon6 A/T). β thalassemia patients were chosen on the basis of codon 39(C/T) non-sens mutation heterozygotie. All subjects had hyperbilirubinemia and 87 of them suffered from cholelthiasis. Demographic, hematological and clinical data of all studied subjects are summarized in table 1.

Methods

Clinical events analyzed

Liver/biliary ultrasound scans were performed annually to detect cholelithiasis in patients over the age of three years. Cholelithiasis was diagnosed on the basis of echodense images within the gall bladder with acoustic shadowing or gravitational change in position.

Laboratory methods

Hematologic data were obtained with an automated cell counter (ABX pentra 60c+). Diagnosis of sickle cell anemia and β thalassemia was performed using cation-exchange high performance liquid chromatography (HPLC) and further confirmed by means of molecular methods. Genomic DNA was extracted from peripheral blood by the standard phenol-chloroform procedure. Mutation at codon6 of βS globin gene was determined by restriction fragment length polymorphism (RFLP) using DdeI as described by Romana et al 2000[12]. Mutation at codon 39 of βS globin gene was determined by PCR/sequencing as described by Chouk et al 2004[12]. Biochemical data were averaged for each patient in steady state (at least three values). Total and fetal hemoglobin (HbF) concentrations, reticulocyte count and other hematologic parameters were determined. Total, unconjugated and conjugated bilirubin concentrations were determined in serum by a standardized colorimetric procedure using automate Konelab 20.

A (TA)nTAA genotyping

A (TA)nTAA sequences were genotyped by polymerase chain reaction (PCR) using a couple of primers namely TAF: 5'TCGTCCTTCTTCCTCTCTGG3' and TAR: 5' TCCTGCTCCTGCCAGAGGTT3'. Polymerase chain reaction was performed in 25µl reaction volumes containing 100ng genomic DNA, 0.2mmol/l of each dNTP, 50mmol/l KCl, 15mmol/l Tris–HCl PH 8.0, 2.5mmol/l MgCl2, 0.5U Amplitaq polymerase (Invitrogen life technologies, Carlsbad,CA, USA) and 10pmol of each forward and reverse primers. The PCR cycling conditions included an initial denaturation step of 10mn at 96°c followed by 35 cycles of 96°C for 30s, annealing at 58°C for 30s and extension at 72°C for 1min. The run was ended by a final extension at 72°C for 7min.

PCR products were than purified and doubly sequenced (forward and reverse) by ABI PRISM Big Dye Termination ready reaction kit and an ABI 310 DNA sequencer (Applied Biosystems, Foster City, USA). The sequences obtained were analyzed using BioEdit software (v 7.0).

Statistical analyses

The demographic and hematologic data are normally distributed, so we used means and standard deviations. The bilirubin data are not normally distributed, so we used medians. For each variable (demographic, hematological and biochemical) difference between cases and controls were evaluated applying the t test or the nonparametric Mann-Whitney test as appropriate using SPSS (version 18). Hardy-Weinberg equilibrium was tested using the software package Arlequin (version 3.01). Genetic differences between cases and controls were evaluated applying exacts tests to genotypic or allelic contingency tables using compare 2 (version 1.02). The relationships between genotypes found and total bilirubin level was evaluated applying Fisher's exact test using compare 2 (version 1.02). We calculated p values for entire tests and Fisher's exact test and chi-squared test was used as appropriate.

RESULTS

Demographic, hematological and biochemical analysis

The analysis of the demographic parameters between the different groups of patients shows a significant association with age. The risk factor of developing cholelithiasis is more important among younger patients (p>0.05) (Table1).

The distribution of each continuous variable was performed using the nonparametric Mann-Whitney test. Our results show that there is no significant difference between the two groups of SCA patient according to the presence or the absence of cholelithiasis (p>0.05) (Table1). Whereas, the comparison of total, conjugated and unconjugated

Table 1 : Hematological, demographic and clinical data of studied population

	βthalassemia patients with cholelithiasis	βthalassemia patients without cholelithiasis	p	SCA adult patients with cholelithiasis	SCA adult patients without cholelithiasis	p
Number	50	26		47 SS	28SS	
Age (mean ;range)	30; 25-35	20; 15-25	0.049	35; 25-46	21; 18-24	0.03
Sex ratio (M/F)	20/30	10/16	0.520	16/31	10/18	0.352
Hb (g/dl)	9.6±0.9	9.9±0.8	0.842	8.7±0.3	8.8±0.5	0.930
RBC (1012/L)	2.38±1.02	3.22±0.7	0.090	3.29±0.5	3.5±0.2	0.520
MCV (fl)	60±1.3	62±1.02	0.460	79.7±1.3	80.7±0.9	0.530
MCH (pg)	20.3±2.2	24.6±1.8	0.120	34.9±0.7	27.8±1.02	0.850
RDW (%)	8±0.9	7±1.2	0.220	5.83±0.5	6±0.7	0.360
HbA	94.6±0.4	95±0.2	1	0	0	1
HbS (%)	0	0	1	86±0.5	85.8±0.2	1
HbF(%)	0	0	1	11±0.5	11.3±0.2	1
HbA2	5.4±0.4	5±0.2	1	3±0.5	2.9±0.1	1
Total bilirubin level (µmol/l)	86.5	50.04	0.023	73.5	40.51	0.015
Unconjugated bilirubin level (µmol/l)	80.8	37.2	0.011	58.2	20.21	0.012
Conjugated bilirubin level (µmol/l)	5.7	13.2	0.034	15.3	20.3	0.05

Usual value of total bilirubin level is <17µmol/l.
SS: homozygous of - globin gene mutation.
The demographic and hematologic values are indicated as mean ± standard deviation.
The bilirubin values are indicated as medians.
Hb: hemoglobin, RBC: red blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin and RDW: red blood distribution width.
Statistics for the comparison of demographic and hematological variables between the two groups were performed using the t test and chi-square test as appropriate (SPSS 18.0).
Statistics for the comparison of bilirubin level between the two groups were performed using the nonparametric Mann-Whitney test (SPSS 18.0).

bilirubin concentrations between the two groups of patients according to the presence or the absence of cholelithiasis shows a significant difference with p<0.05 (Table1).

Analysis of the A(TA)nTAA polymorphism

All samples were found to be in Hardy-Weinberg equilibrium (p>0.05). Analysis of the A(TA)nTAA polymorphism at UGT1A1 promoter showed the presence of seven different genotypes, namely (TA)6/(TA)6, (TA)6/(TA)5 (TA)6/(TA)7, (TA)5/(TA)7, (TA)7/(TA)7, (TA)7/(TA)8 and (TA)8/(TA)8 within the patient groups. The distribution of genotypes is shown in table 2 and table 3.

In order to search for any association between genotypes and bilirubin level, two intervals of total bilirubin were considered. The first one included interval between 15 and 34 µmol/l, the second from 35 to 90µmol/l. The choice of 35 µmol/l as the cutting point of bilirubin total concentration is based on its relationship with gilbert syndrome associated with genotype (TA)7/(TA)7. As shown in table 4, our results indicated that total bilirubin level was significantly increased in patients carrying (TA)7 or (TA)8 variants, namely (TA)6/(TA)7,(TA)7/(TA)7, (TA)7/(TA)8 and (TA)8/(TA)8. The distribution of genotypes was analyzed according to the presence or absence of gallstones. Statistical analyses showed a significant association of the genotypes (TA)6/(TA)7 and (TA)7/(TA)7 with the presence of gallstone in SCA and β thalassemia groups (Tables 2-3). At the allelic analysis, (TA)7 was found to be associated with cholelithiasis in both β thalassemia and SCA patients with p=7.10-4 , RR = 2.567 (1.44-4.58) and p= 3.6 10-6 with RR = 2.99 (1.832-4.862), respectively.

Table 2 : Distribution of (TA) n genotypes and allele frequency among β minor patients.

	β patients with cholelithiasis. n=50	β patients without cholelithiasis n=26	p	OR CI: 95%
(TA)6/(TA)6	10	16	1*	-
(TA)6/(TA)5	0	1	1	-
(TA)6/(TA)7	25	7	5.110-3	2.813 (1.37-5.18)
(TA)7/(TA)7	10	2	0.015	3.692 (1.01-13.5)
(TA)5/(TA)7	1	0	1	-
(TA)7/(TA)8	3	0		
(TA)8/(TA)8	1	0	1	-
Allele frequency				
(TA)6	0.45	0.77	1*	-
(TA)5	0.01	0.00	1	-
(TA)7	0.49	0.21	7.10-4	2.567 (1.44-4.58)
(TA)8	0.05	0.00	0.217	-

1*: reference group.

Table 3 : Distribution of (TA)n genotypes and allele frequency among SCA patients.

	SCA patients with cholelithiasis n=47	SCA patients without cholelithiasis n=27	P	OR CI: 95%
(TA)6/(TA)6	10	16	1*	-
(TA)6/(TA)5	0	1	1	-
(TA)6/(TA)7	26	9	0.011	2.393(1.26-4.54)
(TA)7/(TA)7	8	1	0.018	5.538(0.85-36.03)
(TA)5/(TA)7	1	0	1	-
(TA)7/(TA)8	2	0	0.553	-
(TA)8/(TA)8	0	0	1	-
Allele frequency				
-(TA)6	0.53	0.71	1*	-
(TA)5	0.01	0.01	0.321	-
(TA)7	0.43	0.19	3.6 10-6	2.99 (1.832-4.862)
(TA)8	0.02	0	0.179	-

Table 4 : Repartition of (TA)n genotypes according to the bilirubin level.

	Group 1	Group 2	P RR(CI95%)
(TA)n genotypes	2	0	1
(TA)6/(TA)5	1	1	0.037 2(0.66-∞)
(TA)5/(TA)7	52	0	1*
(TA)6/(TA)6	7	60	3.310-9
(TA)6/(TA)7	0	22	9.571
(TA)7/(TA)7			(4.75-19.29)
(TA)7/(TA)8	0	5	2.710^{-19} ∞ (145.54-∞)
(TA)8/(TA)8	0	1	2.410-7 ∞ (27.87-∞)
Total	62	89	0.37 ∞ (2.06-∞)

Group 1: total bilirubin level comprising between 15 and 34.
Group 2: total bilirubin level comprising between 35 and 90µmol/l.
1*: reference group. RR: relative risk. CI: confidence interval. ∞: infinity.

DISCUSSION

β hemoglobinopathy is characterized by chronic hemolysis which can lead to the formation of pigment gallstone type. Sickle cell patients and β thalassemia patients who carry the allele (TA)7 are favorable for the Gallstone formation [14]. In two previous studies we have been reported the association of (TA)7 and (TA)8 with cholelithiasis among tunisian patients out of hemolytic disease and in SCA children patients [10,11]. In the current study, we analyzed the implication of the polymorphism A(TA)nTAA in the occurrence of hyperbilirubinemia and gallstones in SCA and β thalassemia patients. Several studies have previously reported that SCA patients with the (TA)7/(TA)7 genotype, associated with increased level of total bilirubin, had a significantly increased incidence of cholecystectomy [15-17]. Similar results were observed in β thalassemia including minor, intermedia and major form [18-21]. Our results show that total bilirubin level is significantly increased among patients with the genotypes (TA)6/(TA)7, (TA)7/(TA)7, (TA)7/(TA)8 and (TA)8/(TA)8. When SCA and β thalassemia patients were stratified according to the presence or absence of gallstones, we showed that the risk of cholelithiasis was significantly increased in SCA subjects carrying the genotype (TA)6/(TA)7 or (TA)7/(TA)7. Interestingly, (TA)7/(TA)7 genotype has been reported to be associated with higher risk of gallstones formation in SCA patients from several countries [12]. However, in a guadeloupe population, an association of cholelithiasis with genotype (TA)7/(TA)8 has been demonstrated by Chaar V et al. 2005. This association is not reported in our population. Although, other reports tested the effect of polymorphism at the promoter of UGT1A1 gene in β thalassemia patients. In fact, in Italy, Galanello R et al (1999) demonstrated that patients with thalassemia major and those with thalassemia intermedia carrying (TA)7/(TA)7 genotype had significantly higher bilirubin levels than in those with (TA)6/(TA)7 or (TA)6/(TA)6 genotype [22]. More recently, studies have showed that the incidence of cholelithiasis was higher in patients homozygous for TA7 [19,23]. Inconsistently, we found a significant association between patients homozygous and heterozygous for TA7 and cholelithiasis (OR=3.692, OR=2.813, respectively).

In conclusion, our data provide evidence that genotypes (TA)6/(TA)7 and (TA)7/(TA)7 represent a risk factor for gallstones formation in SCA and β thalassemia (minor) patients in Tunisia. Accordingly, (TA)6/(TA)7 and (TA)7/(TA)7 has been associated with lower UGT1A1 activity leading to hyperbilirubinemia and subsequently to gallstones formation [5]. The presence of (TA)7 variant of UGT1A1 gene confer severity in the disease independently from β gene mutations. Thus, in patients positive carrying the (TA)7 variant, it could be interesting to act before the formation of cholelithiasis.

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