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ORIGINAL ARTICLE

Early modification of sickle cell disease clinical course by UDP-glucuronosyltransferase 1A1 gene promoter polymorphism

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Abstract Elevated erythrocyte destruction in sickle cell disease (SCD) results in chronic hyperbilirubinaemia and, in a subset of patients, cholelithiasis occurs. We investigated whether the $(TA)_n$ promoter polymorphism in the UDP-glucuronosyltransferase 1A1 gene (*UGT1A1*) may modify bilirubin metabolism, influencing bilirubinaemia, predisposition to cholelithiasis and subsequent cholecystectomy, in a group of 153 young SCD patients (mean age 12.0 ± 9.0 years) predominantly of Bantu beta S haplotype. The concomitant effect of alpha thalassaemia was also analysed. Among the several *UGT1A1* genotypes found, the most frequent were the $(TA)_6/(TA)_6$ ($n = 37$), $(TA)_6/(TA)_7$ ($n = 60$) and $(TA)_7/(TA)_7$ ($n = 29$). These groups of patients did not significantly differ in age, gender ratio and haemoglobin, foetal haemoglobin and reticulocyte levels. On the other hand, total bilirubin levels were significantly different between groups, with an increased (TA) repeat number being associated with higher bilirubinaemia. Furthermore, both cholelithiasis and cholecystectomy were more frequent in groups with higher (TA) repeat number, although the former association was not statistically significant. None of the mentioned parameters is statistically different within *UGT1A1* groups with the presence of alpha thalassaemia. Thus, the *UGT1A1* promoter polymorphism may represent an important nonglobin genetic modifier of Bantu SCD patients' clinical manifestations, even at a young age.

Keywords UGT1A1 · Sickle cell disease · Modifier genes · Hyperbilirubinaemia · Alpha thalassaemia

Introduction

Sickle cell disease (SCD; OMIM #603903) is the most common genetic abnormality affecting people of African

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ancestry. It is caused by a molecular defect in the haemoglobin (Hb) beta (β)-chain, where valine is substituted for glutamic acid in the sixth amino acid position. The resulting variant is called Hb S as opposed to the normal adult Hb A. The polymerisation of deoxygenated Hb S is the primary event in the molecular pathogenesis of SCD, resulting in a distortion of the red cell shape and a marked decrease in its deformability. SCD is characterised by a chronic haemolytic anaemia and recurrent vasoocclusive episodes, leading to multisystemic complications with a wide variation in severity (Serjeant 2001). Part of this clinical heterogeneity can be explained by the coinheritance of genetic modifiers linked or nonlinked to the globin gene clusters (Steinberg and Adewoye 2006).

It is known that the accelerated erythrocyte destruction in SCD often leads to chronic hyperbilirubinaemia, as bilirubin catabolism is the final step in the breakdown of haem from haemoglobin. Consequently, some clinical complications, such as cholelithiasis, recurrent painful abdominal events and cholecystitis are frequent in this pathology.

The primary bilirubin-catabolising hepatic enzyme, UDP-glucuronosyltransferase 1A1 (UGT1A1; Chr 2q37; EC 2.4.1.17), mediates the conjugation of bilirubin into a water-soluble conjugated form that is excreted in the bile. The *UGT1A1* promoter contains an $A(TA)_nTAA$ sequence with a common $(TA)_6$ tandem repeat. The homozygosity for the $(TA)_7$ repeat has been associated with decreased enzyme function and Gilbert's syndrome of unconjugated hyperbilirubinaemia (Monaghan et al 1996). Other alleles, a shorter $(TA)_5$ and a longer $(TA)_8$, have been described in people of African descent (Beutler et al. 1998). Cholelithiasis, by promoting cholecystitis, is responsible for high levels of morbidity in SCD patients, and elective cholecystectomy is therefore recommended for patients developing this complication.

The aim of this study was to determine whether the promoter *UGT1A1* polymorphism (resulting in UGT1A1 activity variation) could modify bilirubin metabolism and affect serum bilirubin concentration in young SCD patients, thereby influencing the development of cholelithiasis and subsequently cholecystectomy. Also, the effect of alpha thalassaemia (α-thalassaemia) within *UGT1A1* genotype groups was investigated.

Patients and methods

This study was performed on 153 young Portuguese SCD patients (mean age 12.0 ± 9.0 years), the majority descending from African immigrants (preponderantly from Angola, Sao Tome and Principe and Cape Verde). All patients, or their legal representatives, gave informed consent prior to their inclusion in the study. SCD steady-state patient data, concerning haemoglobin, foetal haemoglobin, reticulocytes and total bilirubin levels (determined by standard procedures) were averaged for each patient, and clinical information was gathered for cholelithiasis (detected by liver/biliary ultrasound scans) and cholecystectomy. Genomic DNA was extracted from peripheral blood leukocytes by a salting-out procedure (Miller et al 1988). The homozygosity for the SCD mutation was confirmed by a bidirectional allele-specific polymerase chain reaction (PCR) as described (Liu et al. 1997). The number of (TA) repeats in the *UGT1A1* promoter was analysed by a PCR-based methodology using the primers described by Monaghan et al. (1996) in which the sense primer was 5-carboxyfluorescein (FAM) fluorescent-tagged. One aliquot of each PCR product was analysed by Gene Scan 3.7 software, along with a molecular weight marker.

Beta globin (β-globin) gene-cluster haplotypes were assigned after examining nine restriction endonuclease sites within the cluster [*Hin*c II (ε), *Xmn* I (5′ to $^{G}\gamma$), *Hin*d III (within $^{G}\gamma$ and $^{A}\gamma$), *Hin*c II (within and 3′ to $\psi\beta$), *Hin*f I (5′ to β), *Ava* II (within β), and *Hin*f I (3' to β)]. Aliquots of the amplified products were digested with the appropriate restriction enzymes under the conditions recommended by the manufacturer. Haplotypes were established by determining the presence or absence of cleavage at each site and by compiling the results into one pattern classified according to Orkin et al. (1982). The allelic phase was determined by family studies whenever necessary. The $-\alpha^{3.7}$ kb deletion was searched for by gap PCR, as previously described (Dodé et al. 1992). Individuals were scored according to the presence/absence of deletional alleles. In order to perform statistical analyses, SCD patients presenting one or two deletional ($-\alpha^{3.7}$) alleles were integrated in the same group, named α-thal (see Table 3).

All statistical analyses were performed using the statistical software package SPSS 14.0. The significance of the differences between groups of patients was assessed using the Kruskal–Wallis, chi-square, analysis of variance (ANOVA), Wald, Mann–Whitney *U* or Fisher's exact tests, as appropriate. The odds ratios (OR) were obtained by applying a logistic regression model using the *UGT1A1* genotype as the independent variable and the cholelithiasis and cholecystectomy as dependent variables. The reference class was the 6/6 *UGT1A1* genotype. Significance for statistical analysis was defined as a $P < 0.05$.

Results

The homozygosity for the sickle cell mutation (HBB:c.20A > T) was confirmed in all patients. The

UGT1A1 PCR fragments obtained ranged between 96 and 102 bp, which corresponded to a $(TA)_{5–8}$ variation. Consequently, several *UGT1A1* genotypes were found: 5/5 ($n = 1$); 5/6 ($n = 9$); 5/7 ($n = 5$); 5/8 ($n = 3$); 6/6 ($n = 37$); 6/7 ($n = 60$); 6/8 ($n = 6$); 7/7 ($n = 29$) and (7/8) ($n = 3$); (Appendix 1 of Supplementary Material). Only patients belonging to the three largest genotype groups (namely, 6/6, 6/7 and 7/7) were considered for statistical analyses. Comparisons performed for these three groups of patients did not reveal any significant difference concerning age, gender ratio, and Hb, Hb F and reticulocyte levels, which denotes a similar degree of haemolysis between groups. However, total bilirubin levels were significantly different between groups ($P = 0.003$; Kruskal–Wallis test), with an increased (TA) repeat number being associated with higher bilirubinaemia (Table 1).

The chi-square tests used for cholelithiasis and cholecystectomy data showed that both cholelithiasis ($P = 0.407$) and cholecystectomy ($P = 0.016$) were increased in the patients with higher (TA) repeat number, although the former difference was not statistically significant (Table 1). The OR of having cholelithiasis for genotypes 6/7 [OR = 1.500; 95% confidence interval (CI) = 0.595–3.784) and 7/7 (OR = 2.057; 95% CI = 0.708–5.980] was increased when compared with 6/6. The same comparison for cholecystectomy revealed an increased risk only for genotype 7/7 (OR = 4.714; 95% CI = 0.869–25.581) (Table 2).

To gain further insight into the modifying effect of *UGT1A1* polymorphism, the βS haplotype and the α-globin genotype were determined in our SCD patients. The prevalence of the Bantu haplotype was similar among the three *UGT1A1* groups (Bantu allelic frequency in the 6/6 *UGT1A1* group was 73.0%; in the 6/7 group 62.3%; and in the 7/7 group 67.2%; $P = 0.305$). The overall α-thalassaemia ($-\alpha^{3.7}$) allelic frequency was 26% and was not significantly different between *UGT1A1* genotype groups. We found that in young SCD patients predominantly of Bantu βS haplotype, the coinheritance of α-thalassaemia did not significantly influence the level, total bilirubin level, cholelithogenesis and the need for cholecystectomy (Table 3).

Phenotype features of patients belonging to the less frequent genotype groups are summarised in Appendix 1 of Supplementary Material.

Table 1 Comparison of sickle cell disease phenotype features in patients stratified according to *UGT1A1* genotype

	UGT1A1 genotype		
	6/6	6/7	7/7
Number	37	60	29
Age (years)	12.9 ± 8.6	9.0 ± 7.4	12.7 ± 9.0
*P**	0.580		
Gender ratio (M/F)	18/19	30/30	14/15
*P***	0.985		
Haemoglobin (g/dl)	7.88 ± 0.92	7.93 ± 1.37	8.40 ± 1.11
*P****	0.398		
Haemoglobin F (%)	8.33 ± 5.27	10.03 ± 6.39	8.46 ± 5.19
*P**	0.535		
Reticulocytes (%)	10.21 ± 3.98	11.61 ± 6.93	11.70 ± 4.58
*P**	0.500		
Total bilirubin (mg/dl)	2.55 ± 1.78	3.20 ± 3.14	4.67 ± 3.67
*P**	0.003		
Cholelithiasis (%)	29.4	38.5	46.2
*P***	0.407		
Cholecystectomy (%)	5.7	3.8	22.2
*P***	0.016		

* Kruskal–Wallis test

** Chi-square test

*** Analysis of variance test

Discussion

In this study, we investigated the genetic architecture of a young population with a SCD subphenotype characterised by hyperbilirubinaemia and cholelithiasis. The (TA) repeat number found within the *UGT1A1* gene promoter varied between 5 and 8, which was expected due to the African ancestry of this population (Beutler et al. 1998).

The coinheritance of Gilbert's syndrome determinant and genetic alterations associated with disorders that increase turnover of red blood cells or their precursors has been reported to elevate bilirubin levels, e.g. in β-thalassaemia (Galanello et al. 1997, 1999b), G6PD deficiency (Galanello et al. 1999a) and SCD (Adekile et al. 2005; Chaar et al. 2005; Fertrin et al. 2003; Harverfield et al. 2005; Passon et al. 2001). In fact, in addition to the results presented here, the mentioned studies performed in SCD

Table 2 Calculation of odds ratios between *UGT1A1* genotype groups

UGT1A1 genotype	Odds ratios	
	6/6–6/7	6/6–7/7
Cholelithiasis	1.500	2.057
95% CI	0.595–3.784	0.708–5.980
*P**	0.390	0.185
Cholecystectomy	0.647	4.714
95% CI	0.087–4.821	0.869–25.581
*P**	0.671	0.072

CI confidence interval

* Wald test

Table 3 Influence of α-thalassaemia (α-thal.) on bilirubin-associated parameters among *UGT1A1* genotype groups

		UGT1A1 genotype groups		
		6/6	6/7	7/7
Number	αα/αα	19	36	14
	α-thal.	18	24	15
Haemoglobin (g/dl)	αα/αα	7.59 ± 0.87	8.09 ± 1.46	7.70 ± 0.84
	α-thal.	8.14 ± 1.08	7.87 ± 0.93	8.35 ± 1.05
*P**		0.192	0.389	0.155
Total bilirubin (mg/dl)	αα/αα	2.80 ± 2.45	3.61 ± 3.75	5.42 ± 4.94
	α-thal.	2.79 ± 2.41	2.51 ± 1.53	3.98 ± 1.84
*P**		0.961	0.205	0.793
Cholelithiasis (%)	αα/αα	29.4	41.2	58.3
	α-thal.	29.4	36.8	35.7
*P***		1.000	1.000	0.431
Cholecystectomy (%)	αα/αα	11.8	5.7	30.8
	α-thal.	0.00	0.00	14.3
*P***		0.229	0.535	0.385

* Mann–Whitney test

** Fisher's exact test

patients have shown that homozygosity for the $(TA)_7$ *UGT1A1* genotype significantly influences bilirubin metabolism and is associated with higher steady-state concentrations of unconjugated serum bilirubin. At least in our case, this fact could not be explained by increased haemolysis, as there are no significant differences in average Hb concentration and in reticulocyte count among the different *UGT1A1* genotype groups (Table 1). Some other studies were performed regarding the influence of the *UGT1A1* polymorphism on cholelithiasis, but the association has been less consistent that with a more proximal phenotype, namely, hyperbilirubinaemia. Moreover, age has been considered a modifier of *UGT1A1* polymorphism influence. As an example, Harverfield et al. (2005) observed that the $(TA)_7$ homozygosity is a significant risk factor for cholelithiasis development only in older SCD patients (34.6 ± 10.9 years) but not in a younger adult cohort group (25.0 ± 2.9 years). Although gallstones were identified at a mean age of 15.1 years in 40.6% of this cohort group, there was no association with *UGT1A1* $(TA)_n$ genotypes (Harverfield et al. 2005). On the other hand, Fertrin et al. (2003) analysed a group of adult SCD patients (28.5 ± 10.7 years) and observed that although the frequency of cholelithiasis was higher in patients with the 7/7 genotype, the difference was not statistically significant (Fertrin et al. 2003). Considering the need for cholecystectomy, the difference between 6/7 and 6/6 patients was statistically significant, although the difference between the other groups was not (Fertrin et al. 2003). Furthermore, in studies performed in SCD children (3–19 years) the development of symptomatic cholelithiasis requiring cholecystectomy was significantly higher for children with the 7/7 genotype than those with the 6/7 or 6/6 genotypes (Passon et al. 2001). Finally, Chaar et al. (2005) observed that the frequency of cholelithiasis was significantly higher in an SCD group of children having 7/7 or 7/8 genotype. On the whole, analysing our results along with previously published data, we can conclude that the *UGT1A1* promoter genotype is unquestionably an important genetic determinant of hyperbilirubinaemia in SCD. However, its influence on cholelithiasis development is not so straightforward and seems to be modulated by other factors, among which age is relevant. Concerning patients who need cholecystectomy, their inclusion in the 7/7 genotype group is remarkable, thereby confirming the penetrance of this genotype.

It is known that the coinheritance of α-thalassaemia and SCD determinants reduces the mean corpuscular haemoglobin concentration of red blood cells, which tends to inhibit sickling. It was observed that the coexistence of α-thalassaemia decreases the chance of developing gallstones in Arab Indian haplotype SCD patients, which may be related to lower haemolysis, as suggested by their predisposition to present, in general, higher mean Hb levels (Haider et al. 1998). In addition, Adekile et al. (2005) observed that the *β*-globin haplotype and the coexisting α-thalassaemia did not have significant influence on serum bilirubin levels in Arab Indian haplotype SCD patients (Adekile et al. 2005). Furthermore, Chaar et al. (2006) showed that although α-thalassaemia is associated with a modest reduction in haemolysis and unconjugated bilirubin level, *UGT1A1* polymorphism outweighs its effect on cholelithogenesis in SCD patients predominantly of Benim *β*S haplotype (Chaar et al. 2006). Vasavda et al. (2007) also observed that α-thalassaemia was significantly associated with reduced bilirubin levels in SCD adult patients. In our young SCD patient series predominantly of Bantu *β*S haplotype, we found that the coinheritance of

α-thalassaemia does not significantly influence Hb level, total bilirubin level, cholelithogenesis and the need for cholecystectomy (Table 3). However, we observed that the coinheritance of α-thalassaemia is slightly associated with a positive effect on the mentioned parameters, mainly in the 7/7 genotype group in which patients presented higher haemoglobin level, lower bilirubin, fewer cholelithiasis and undergo cholecystectomy less frequently.

In conclusion, our study, the first performed regarding young Bantu SCD patients, reinforces the concept that the sickle cell mutation alone is not sufficient to explain the wide range of phenotypic expression characteristic of SCD. Our results attribute to *UGT1A1* promoter polymorphism an important modifying effect of the clinical manifestations in Bantu SCD patients, namely, in bilirubin metabolism, cholelithiasis development and the need for cholecystectomy, even at a young age. Also, we observed that in these patients, the modulating effect of the *UGT1A1* promoter polymorphism is not significantly altered by the coinheritance of α-thalassaemia determinants.

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