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DOES BILIRUBIN LEVEL CORRESPOND TO INTERACTION OF c.-3279T>G AND A(TA)7TAA VARIANTS IN UGT1A1 GENE?

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Abstract – Promoter variants c.-3279T>G and A(TA)7TAA show decreased level of expression of UDP-glucuronosyl transferase 1A1 (UGT1A1) and consequently reduced activity of the enzyme catalyzing glucuronidation of bilirubin in hepatocytes. Thus, coincidental occurence of both variants should lead to increase of hyperbilirubinemia or contribute to its manifestation. In this study, investigation of both variants in 101 patients and 84 controls in a Caucasian population was performed and the results were compared with serum bilirubin levels. Despite high linkage disequilibrium between the loci (D´ = 0.91, r^2 = 0.69), we have proven an interaction between the variants increasing the odds ratio for [(TA)7]+c.[-3279T>G] homozygotes to 54.2.

Key words: c.-3279T>G, A(TA)7TAA, UGT1A1, Gilbert´s syndrome, bilirubin

INTRODUCTION

UGT1A1 catalyzes glucuronidation of bilirubin and other xenobiotics in hepatocytes and in this way it participates in heme degradation. The most frequent variant in the Caucasian population, two base insertion of TA nucleotides in TATAA box of UGT1A1 gene and its association with Gilbert´s syndrome, was firstly described in 1995 by Bosma *et al.* TATAA box region is the binding site of transcription factor IID, which iniciates the transcription. In vitro experiments revealed decreased transcription and reduced enzymatic activity in altered [(TA)7] allele of UGT1A1 up to 10-30% (1, 3, 4, 7, 18).
Impaired glucuronidation results in Gilbert´s syndrome - mild unconjugated hyper-bilirubinemia with fluctuating bilirubin levels from 20 to 50 µmol/l (rarely up to 100 µmol/l).

Abbreviations: **PBREM,** phenobarbital enhancer module; **UGT1A1**, UDP-glucuronosyl transferase 1A1; **A(TA)7TAA**, (TA)7; **OR**, odds ratio.

The frequency of altered [(TA7)] allele among the Caucasian population is 30-40%, resulting in the homozygous Gilbert´s genotype (TA)7/(TA)7 in 9-16% of them. Since only about 5% develops into Gilbert´s syndrome, other factors contributing to the hyperbilirubinemia development are necessary (3, 14, 17, 18).

Other promoter variant c.-3279T>G is located in the phenobarbital responsive enhancer module (PBREM) of the gene. This 51bp enhancer is regulated by the transcription factor constitutive active receptor in response to phenobarbital induction (8). As well as in the previous variant, the reduced activity of UGT1A1 in phenobarbital enhancer variant c.-3279T>G was shown experimentally (20). The coincidental occurence of more genetic defects and/or the effects of epidemiologic factors such as stress, infection, starvation, is supposed to be the cause of the manifestation of hyperbilirubinemia. The relation of more genetic defects and hyperbilirubinemia was well documented in the Japanese population in the most common change p.Gly71Arg and (TA)7 variant.

Also, a haplotype analysis of UGT1A1 gene in the Korean population was published, but distribution of UGT1A1 variants in the Caucasian population differs (12, 13, 20).

Maruo *et al* postulated that coincidental occurence of homozygous variants [(TA)7]+c.[-3279T>G] is the principal cause in development of Gilbert´s syndrome. However, Jirsa *et al* did not confirm this theory. We believe that either genetic or environmental factors can contribute to the development of hyperbilirubinemia. Then in vitro studies can not simulate in vivo bilirubin metabolism conditions properly and we should also be aware of individual differences between bilirubin metabolism in men and women. As we know, no correlation of bilirubin, PBREM and TATAA alterations in UGT1A1 was carried out in probands and controls (10, 11).

MATERIALS AND METHODS

(TA)7 and c.-3279T>G variants were investigated in 101 patients and 84 controls from a Caucasian population. Blood samples were taken with the signed informed consent of the participants. Genomic DNA was extracted from the peripheral blood leukocytes according to the standard procedures. Genotyping of TA repeats of UGT1A1 gene was performed by PCR and the sequencing with 5´-AACTCCCTGCTACCTTTGTGG-3´forward and 5´-TCAACAGTATCTTCCCAGCATGGG-3´reverse primers (product size 239 bp). Analysis of c.-3279T>G promoter variant was carried out by PCR and RFLP with the use of *Hpy8I* restrictase (10). In all subjects, liver tests were checked (total serum bilirubin and its conjugated fraction, ALT, AST). Statistic analysis was performed by CubeX and odds ratio calculator (2, 6).

RESULTS

Criteria for probands group were determined according to the guidelines for Gilbert´s syndrome diagnostic – mild unconjugated hyperbilirubinemia with a lower limit of bilirubin 17 µmol/l without hemolysis or other liver injury. Control and proband groups were consistent in sex and age; the average age of controls was 16 years (SD±4.19) and in probands 17 years (SD±5.3). Average total serum bilirubin level in controls was 10.69 µmol/l (SD±4.0) compared with 46.7 µmol/l (SD±26) in probands.

Frequency of wild type [(TA)6] allele in controls was 67.86% versus [(TA)7] allele 32.14 %. Further frequency of allele T in c.-3279T>G variant was 61.31 % in contrast to allele G 38.69 % in controls. Linkage between alleles [(TA)7] and c.[-3279T>G] was D´= 0.79, r^2 = 0.47 in controls. No homozygote for [(TA)7]+c.[-3279G] was found in controls. However, two homozygotes (TA)7/(TA)7 was heterozygous for c.-3279T>G (see Table 1). Correlation of c.-3279 T>G and (TA)7 genotype and bilirubin levels was evident in (TA)6/(TA)7 heterozygotes, however the difference was not significant (see Table 2).

In the probands group (n = 101), allele [(TA)7] frequency was 97%, in the remaining 3% wild type was presented. Also, the wild type allele c.[-3279T] was in a minority (0.99%) in comparison with 99.01% frequency of allele c.[-3279T>G.] All wild type alleles in probands were presented in a heterozygous state. Linkage disequilibrium in probands expressed with D´was high (D´ = 1), but r^2 was very low (r^2 = 0.32). Correlation of genotype and bilirubin levels was not possible to carry out because of the uniformity of genotypes in this group.

Table 1. Distribution of genotypes in probands and controls

	Probands (n = 101)				Controls (n = 84)		
	T/T	T/G	G/G		T/T	T/G	G/G
6/6	-	-	-	6/6	25	6	1
6/7	-	2	4	6/7	4	37	9
7/7	-	-	95	7/7	-	2	0

Table 2. Average of bilirubin levels according to genotype in controls (in µmol/l)

	T/T	T/G	G/G
6/6	8,37 (±2,31)	8,47 (±0,81)	13
6/7	8,48 (±3,66)	10,58 (±4,39)	13,62 (±3,15)
7/7	0	12,3 (±4,53)	0

The overall survey of proband and control group was as follows. There is no doubt about the higher frequency of [(TA)7] allele in probands (97%) than in controls (32.14%) as well as the 99.01% frequency of c.[-3279T>G.] allele in probands compared with 38,69 % frequency of the same allele in controls. Linkage disequilibrium of two variants in both groups was significant D´= 0.91, r^2 = 0.69 (P<0.0001). The distribution of genotypes for the haplotypes formed by these 2 loci is significantly different between patients and controls – P = 0.00005, chí = 24.99, df = 4. The odds ratio (OR) for the risk allele c.[-3279T>G.] in the PBREM is 34.42 (95% confidence interval from 19.14 to 61.89), and 38.71 for the risk allele [(TA)7] in the TATAA box (95% confidence interval from 21.71 to 68.99). The odds ratio increases for the

homozygotes for both risk alleles to 54.20 (95% confidence interval from 30.15 to 97.45).

DISCUSSION

The aim of our study was to establish the role of c.-3279T>G on hyperbilirubinemia in humans. We have demonstrated that this variant is strongly associated with affection by the Gilbert's syndrome, with OR 34.42. This association signal is probably not only due to strong linkage disequilibrum with the other variant (TA)7, because we have shown additive efect of these two variants by demonstrating an increase of the OR for the homozygotes for both variants. We correlated bilirubin levels with coincidental occurence of both variants. Regular data collection and limit estimation is important in epidemiological studies and should not be underestimated. In Gilbert´s syndrome, different results can be obtained with diverse criteria. Since the occurence of (TA)7 allele in the Causcian population is 30-40%, we cannot reject controls with (TA)7/(TA)7 with no signs of hyperbilirubinemia. Also the variability of bilirubin in an individual can influence the results, which is why we calculated with average bilirubin level obtained from at least three measurements.

In bilirubin-genotype comparison the most interesting was the effect of c.-3279T>G variant in conjuction with the (TA)6/(TA)7 in controls. In these individuals, the bilirubin levels were the following: (in µmol/l): 8.48 in PBREM wild type T/T; 10.58 in heterozygotes T/G and 13.62 in homozygotes G/G. This indicates the increasing tendency of bilirubin according to the PBREM genotype and which is also apparent the in (TA)6/(TA)6 controls (see Table 2). However, these results has not been found significant, probably due to the low number of examined controls. Bilirubin levelsin (TA)7 variant has been documented many times. In this study, the average serum bilirubin in probands with both homozygous variants (TA)7 and c.-3279T>G was 46.7 µmol/l (SD±26). The high SD of bilirubin level in probands is caused by the presence of about five individuals with bilirubin over 100 µmol/l in probands group. In these five probands, no defect in the structural region of UGT1A1 was found.

Results from both groups contained all genotypes with exception of homozygote (TA)7 together with homozygote wild type PBREM allele c.-3279T. However, according to high linkage disequilibrium D´=0.95 allele (TA)7 is linked with c.-3279T>G allele, this and other studies document genotype (TA)6/(TA)7 together with c-3279T>G/-3279T>G and even (TA)6/(TA)6 coincidentally with c-3279T>G/-3279T>G (data calculated from Jirsa *et al*) (10). The most interesting fact is that we found no homozygote (TA)7 together with homozygote wild type c.-3279T. In no other studies of Caucasians, African-Americans or Japanese with total number of investigated persons more than 800 found this genotype (5, 9, 10, 13). This is remarkable because homozygous wild type (TA)6 together with c.-3279T>G allelic variant does exist. However, controversially the homozygous wild type -3279T together with mutated homozygote (TA)7 does not occur.

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