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Polymorphism of *UGT1A1*28* $(TA)_7$ and liver damage in hepatitis B virus-positive patients in Albania

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ABSTRACT. Hepatitis B virus (HBV) is the infectious agent of both acute and chronic hepatitis. HBV exists in multiple genotypic variants that differ in their capacity to become persistent chronic infections and in their clinical manifestations, including hepatocellular carcinoma. The 8 genotypes (A-H) of HBV show a specific worldwide geographic distribution and are correlated with different disease course, severity, and response to therapy. We isolated DNA from 75 HBV-positive blood donors, chosen randomly from the database of the National Blood Bank in Tirana, to specifically analyze the *UGT1A1* polymorphism to determine its correlations with bilirubin levels and liver function. The large number of subjects who were HBV-positive carriers of

heterozygosis or homozygosis for the *UGT1A1*28* $(TA)_7$ polymorphism suggests that these individuals may be more susceptible to cancer and should follow a strict regime of prevention.

Key words: Bilirubin; Hepatitis B virus; Hepatocellular carcinoma; *UGT1A1* polymorphism

INTRODUCTION

UDP-glucuronosyltransferase (UGT) enzymes comprise a superfamily of key proteins that catalyze the glucuronidation reaction of a wide range of structurally diverse endogenous and exogenous chemicals. The UGT1A1 enzyme is the only relevant enzyme in bilirubin metabolism. At least 113 variants have been identified, very few of which are common in the general population. Polymorphisms in the promoter region of the *UGT1A1* gene are caused by variability in the number of TA repeats in the TATA-box, which is located upstream of *UGT1A1*. The presence of 7 TA repeats (*UGT1A1*28*) is associated with reduced *UGT1A1* expression compared to the wild-type allele (*UGT1A1*1*), which contains 6 TA repeats. Homozygous individuals who carry the A $(TA)_7$ TAA allele show significantly higher plasma levels of unconjugated bilirubin caused by a 30% reduction in the transcription of *UGT1A1* (Fertrin et al., 2003). Interethnic differences have been observed in the frequency of the *UGT1A1*28* allele, which has an approximate incidence of 6-12% in the Caucasian population, 0-3% in the Asian population, and 16-19% in the African population (Zhou et al., 2008). Most genetic alterations in the *UGT1* gene complex described to date are rare mutations associated with 2 severe familial forms of unconjugated hyperbilirubinemia syndromes (Crigler-Najjar type I and II disorders). Functional studies have revealed that increasing the number of repeats in the promoter region leads to a decreased rate of transcription initiation of the *UGT1A1* gene. The wild-type allele (*UGT1A1*1*) contains 6 TA repeats, whereas the most common variant allele (*UGT1A1*28*) contains 7 TA repeats and is associated with a mild form of inherited unconjugated hyperbilirubinemia syndrome (Gilbert's syndrome) (Bosma et al., 1995; Monaghan et al., 1996). In addition to catalyzing endogenous metabolites (such as bilirubin and steroid hormones), this superfamily of enzymes also catalyzes glucuronidation of a wide range of compounds such as therapeutic drugs and different classes of carcinogens (e.g., heterocyclic and polycyclic hydrocarbons and heterocyclic amines). As a result, UGT enzymes play an important role in cell defense and detoxification. Genetic alterations of these enzymes have been identified as independent predictors of liver cancer (Vogel et al., 2001) with the additional role of increasing the risk of carcinogenesis in patients with hepatitis B virus (HBV) (Kong et al., 2008).

Moreover, HBV infection can lead to progressive hepatic diseases, including hepatic cirrhosis and hepatocellular carcinoma, with about one million deaths per year from HBV-associated hepatocellular carcinoma (Kidd-Ljunggren et al., 2004).

HBV exists in multiple genotypic variants that differ in their capacity to become persistent chronic infections and in their clinical manifestations, including cancer. Eight genotypes (A-H) of HBV have been identified; they differ from each other in 4% of nucleotides. These genotypes show a specific worldwide geographic distribution and are therefore important for studying molecular evolutionary models and patterns of distribution. Structural and functional differences between genotypes may influence the course and severity of disease and the probability of complications, seroconversion of antigen e of HBV, and the response to therapeutic drugs and vaccination (Norder et al., 2004).

In this study, we obtained whole blood from 75 anonymous HBV-positive patients, chosen randomly from the database of blood donors in the city of Tirana. We performed hematological analyses related to liver damage. We then optimized a method for identifying HBV genotypes in a pooled sample from 10 individuals; this small sample size was used to verify literature evidence that genotype D is predominant in Albania (Zehender et al., 2012). We then examined the correlation between the *UGT1A1* genotype and liver function, including levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), alkaline phosphatase, albumin, unconjugated bilirubin, and total bilirubin levels.

MATERIAL AND METHODS

Subjects

DNA samples from 75 subjects positive for the antigen HBsAg (HBV+) were chosen anonymously and randomly from the National Blood Bank in Tirana, Albania. DNA was extracted from 0.5 mL whole blood using a kit based on the salting-out method (Blood DNA kit E.N.Z.A., Omega bio-tek, Norcross, GA, USA). Whole blood samples were analyzed for routine hematological data, city of provenance, and gender (male: female ratio 58:17). A summary of hepatic parameters evaluated in the study group is shown in Table 1.

Table 1. Biochemical characteristics of blood donors.

	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	ALB g/dL	Indirect bilirubin mg/dL	Total bilirubin mg/dL
Minimum	13	6	17	4	1.5	0.1	0.1
Mean	50.4	24.7	46.2	22.1	3.3	0.4	0.5
Median	44	20	44	18	3.2	0.4	0.5
Maximum	179	100	100	77	4.9	0.8	1.3
SD	30.2	15.7	17	15.4	0.7	0.2	0.3

ALT = aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GGT = γ-glutamyl transpeptidase; ALB = albumin.

HBV genotyping

To identify HBV genotypes, 10 samples, selected randomly from our population group, were analyzed using a nested polymerase chain reaction (PCR) that was sensitive for the genotyping of integrated HBV in the DNA of leukocytes (Datta et al., 2009). The amplified region contains a sufficient number of sites that vary between the different HBV strains to identify the viral subtype (A-H) using sequencing methods.

Briefly, the first pair of external primers, 5'-ACCCCTGCTCGTGTTACAGGC-3' (primer sense, position 184-204) and 5'-AAAGCCAGACAGTGGGGGAAA-3' (primer antisense, position 731-711), amplified a product of 547-base pairs (bp) in a region of the gene encoding the major loop of the hydrophilic superficial region of the protein HbsAg. A second pair of internal primers, 5'-GACTCGGTGGTGGACTTCTCTC-3' (primer sense, position 251-271) and 5'-TAAACTGAGCCAGGAGAAACG-3' (primer antisense, position 679-659), amplified a product of 429-bp. For the PCR, we used the following thermal profile: 95°C for 5 min of initial denaturation, 95° for 15 s for continuous denaturing, 60°C for 30 s for annealing, 72°C for 40 s for polymerization, followed by a final extension at 72°C for 5 min; this cycle was repeated

38 times for the "external" PCR and 42 times for nested PCR. Each 25-µL reaction mixture contained 30-50 ng DNA, 8 pmol of each primer, 2.5 µL 10X PCR buffer, 0.5 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.75 U *Taq* polymerase (Fermentas, Vilnius, Lithuania). The reactions were carried out in a thermocycler (Esco, Hamburg, Germany). After amplification, 3 µL amplification product was subjected to electrophoresis on a 2% agarose gel stained with Gel Red (Biotium, Hayward, CA, USA) and observed under ultraviolet light. PCR product sizes were estimated by comparison with a 100-bp DNA ladder as a marker (Fermentas). The products of nested PCR were purified using the Cycle Pure Kit (Omega bio-tek) and sequenced using the nested sense and antisense primers separately with the following thermal protocol: 96°C for 20 s denaturing, 55°C for 20 s annealing, and 60°C for 4 min extension repeated for 30 cycles. The reaction mixture was composed of 1 µL dimethyl sulfoxide, 5 µL DTCS Quick Start Master Mix (Beckman Coulter, Brea, CA, USA), 5 µL 1 µM of each sense and antisense primer, and 9 µL amplified PCR product. The product was purified again using a mixture of 4.6 µL Stop Solution (2 µL Na_2-ethylenediaminetetraacetic acid, 2 µL 3 M sodium acetate, and 0.6 µL glycogen) and 60 µL 100% cold ethanol for each sample, precipitated by centrifugation (15 min at 15,000 *g* and 4°C), and washed with 70% ethanol. Once precipitated (2 min at 15,000 *g* and 4°C) and the supernatant was removed, the pellet was resuspended in 40 µL Sample Loading Solution and analyzed in the Beckman Coulter CEQ 8000 automatic sequencer. The sequences produced by the samples were compared with several sequences for genotypes A-H of HBV in GenBank using the BioEdit software (Hall, 1999) and considering 24 sites of variation between genotypes.

UGT1A1 genotyping

We amplified 75 DNA samples in the promoter region of the gene *UGT1A1*. We used the primers UGT1A1_TAF (TACGCATCCCAGTTTGAGACGTCCCTGCTACCTTTGTGGAC), UGT1A1_TAR (AGCAGGCCCAGGACAAGT), and TAG1_6FAM (TACGCATCCCAGTTTGAGACG). The forward primer was marked with a D4 fluorophore. This enabled determination of the dimension of the amplicon and allelic size in the sequencing phase. For each sample, the amplification reaction mixture included the following components (final concentrations): 1X buffer TaqBuf $(NH_4)_2$, SO_4^-, and $MgCl_2$ (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM primers, 0.1 µM UGT1A1_TAF primer, 0.02 U/µL *Taq* DNA Polymerase (Fermentas), 4 ng/µL DNA. The amplification cycle was: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, primer extension at 72°C for 40 s, and final extension at 72°C for 5 min. The amplified samples were analyzed using the Beckman Coulter CEQ 8000 automatic sequencer for capillary electrophoresis coupled with laser detection. Using this method, we calculated the size of each PCR product represented by the height and area of each fluorescent peak. The electropherogram showed 2 peaks separated by 2 nucleotides (206 and 208 bases) for heterozygosis and by 1 peak of 206 bases for homozygosis for the wild-type allele or by 1 peak of 208 bases for wild-type homozygosis.

To each sample, we added an internal reference standard of known size (400-600 bp, GenomeLab DNA Size Standard Kit -400, Beckman Coulter) to enable identification of fragment sizes. A series of 4 dilutions were prepared for each PCR product at 1:2, 1:5, 1:10, and 1:20 to optimize signal intensity. For each dilution, we prepared a mixture containing the marker of known molecular weight and the sample loading solution (Beckman Coulter). The 40-µL mixture contained: 38.5 µL sample loading solution, 1.0 µL diluted PCR product, and 0.5 µL 400-bp ladder. Frag-3 (Beckman Coulter Software) was used to process the electro-

pherograms and analyze the results to determine the size of each amplicon and the wild-type allele $(TA)_6TAA$ from that with the extra repetition $(TA)_7TAA$. For simplicity, the genotypic structure of the subjects is presented as wild-type for homozygosis for $(TA)_6TAA$, mutation for $(TA)_7TAA$, and heterozygote for $(TA)_6TAA/(TA)_7TAA$ in the tables.

Statistical analysis

We investigated the potential associations between bilirubin, liver damage, and genotypic polymorphism of *UGT1A1*28* $(TA)_7$ in the study group. Groups showed differences in liver function based on the range of values obtained for ALT (10-35 U/L), AST (10-35 U/L), and GGT (10-45 U/L); liver function was considered abnormal (Group B) if more than one of the considered parameters was greater than normal ranges. For statistical analysis, we used the χ^2 test (significance level $P < 0.05$) and the analysis of variance with the MedCalc software (Mariakerke, Belgium).

RESULTS

Only 2 of 10 samples analyzed showed the HBV genotype integrated into the DNA of the study group. The 2 subjects in question had the D genotype.

Genotypic analysis of polymorphism of *UGT1A1*28* $(TA)_7$ in the samples showed 29 subjects with wild-type (39%), 38 subjects with heterozygote (51%), and 8 subjects with mutation (11%). Liver parameters are reported in Table 2 regarding the 3 genotypic structures (wild-type, heterozygote, and mutation).

Table 2. Biochemical characteristics of blood donors according to *UGT1A1*28* $(TA)_7$ genotype.

		ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	ALB (g/dL)	Indirect bilirubin (mg/dL)	Total bilirubin (mg/dL)
Genotype								
WT	Minimum	13	9	17	4	1.6	0.2	0.2
	Mean	54.4	26.4	48.2	26.9	3.4	0.4	0.5
	Median	44	20	45	21	3.3	0.4	0.5
	Maximum	170	100	100	77	4.9	0.8	1.3
	SD	35.7	17.5	19	20	0.7	0.15	0.3
HT	Minimum	18	6	22	5	1.5	0.1	0.1
	Mean	43.4	20.7	44.5	19.1	3.2	0.4	0.5
	Median	40.5	19.5	41.5	14.5	3.2	0.4	0.5
	Maximum	89	50	81	47	4.6	0.8	1.1
	SD	17.5	9.8	15.5	11.1	0.7	0.17	0.2
MT	Minimum	34	11	25	8	2.6	0.2	0.2
	Mean	69	37.1	46.6	19.8	3.3	0.5	0.7
	Median	58	31	46.5	19.5	3.2	0.6	0.8
	Maximum	179	83	73	35	4.2	0.8	1.1
	SD	46.7	24	17.2	9.3	0.6	0.22	0.3
ANOVA	F-ratio	2.959	4.253	0.378	2.309	0.432	0.553	2.448
	P value	0.058	0.018	0.687	0.107	0.782	0.577	0.094
Liver function test								
Y group	Wt		HT		MT		TOT	P
A	21 (36.2%)		34 (56.9%)		4 (6.9%)		58	0.0001
B	8 (47%)		5 (29.4%)		4 (23.6%)		17	

ALT = aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GGT = γ-glutamyl transpeptidase; ALB = albumin; WT = wild type; HT = heterozygote; MT = mutant; TOT = total; ANOVA = analysis of variance. Summary of hematological characteristics of the population by dividing per genotype: WT [*UGT1A1*28* (TA) 6/6), HT (*UGT1A1*28* (TA) 6/7], and MT [*UGT1A1*28* (TA) 7/7]. Liver function test: the group division (A and B) was obtained considering the range of normality of ALT (10-35 U/L), AST (10-35 U/L), and GGT (10-45 U/L); liver function was considered abnormal (Group B) if more than 1 of the considered parameters was greater than normal range.

Based on this division by genotype, analysis of variance showed significance for AST (P = 0.018) and non-significance for ALT (P = 0.058), ALP (P = 0.687), GGT (P = 0.107), ALB (0.782), and for indirect bilirubin levels (Figures 1 and 2). Two-way analysis of variance, considering gender as a factor, did not change these results (data not shown). Association analysis (χ^2 test) between genotype and liver damage as the liver function test was very significant (P < 0.0001), but the result may not be reliable because of the very low number of patients in group B (Table 2).

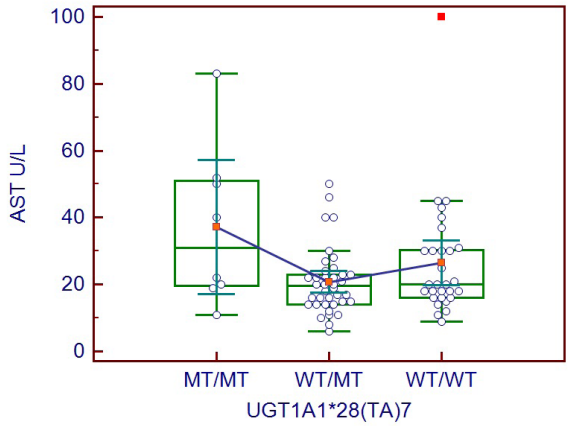

Figure 1. Distribution of *UGT1A1*28* $(TA)_7$ polymorphism compared to aminotransferase (AST) levels. The box plot shows the medians (internal horizontal lines) of AST levels by genotype, the interquartile range (IQR, central box, 25-75th percentile), and the error bars (1.5 x IQR); points represent single samples divided by genotype (MT/MT = 7/7, WT/MT = 6/7, and WT/WT = 6/6), on the x-axis, and by AST levels (U/L) on the y-axis.

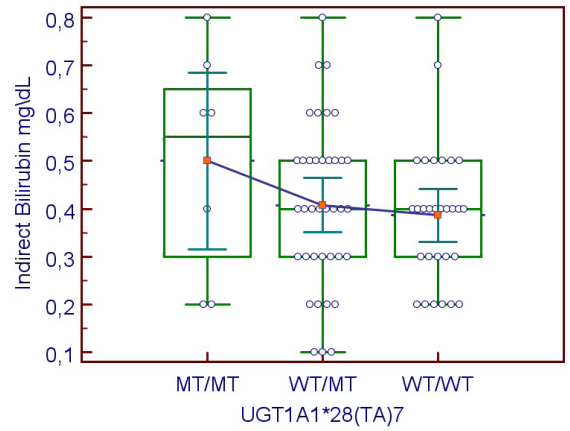

Figure 2. Distribution of *UGT1A1*28* $(TA)_7$ polymorphism with respect to indirect bilirubin levels. MT = mutant; WT = wild type. The box plot shows the medians (internal horizontal lines) of indirect bilirubin levels by genotype, the interquartile range (IQR, central box, 25-75th percentile), and the error bars (1.5 x IQR); points represent single samples divided by genotype (MT/MT = 7/7, WT/MT = 6/7, and WT/WT = 6/6), on the x-axis, and by indirect bilirubin levels (mg/dL) on the y-axis.

DISCUSSION

The aim of this study was to assess the influence of the *UGT1A1* 28* $(TA)_7$ polymorphism on the detoxification activity of the UGT1A1 enzyme by evaluating liver function parameters in blood samples. Our results only revealed a correlation between AST levels and impaired enzyme activity. We did not observe that bilirubin levels were influenced by genotype, a result that is in contrast with other clinical research showing a highly significant relationship between *UGT1A1* (TA)n alleles and bilirubin levels (Haverfield et al., 2005; Lingenhel et al., 2008). Although this trend was observed, the results were not statistically significant, possibly because of the small number of blood donors with the *UGT1A1*28* $(TA)_7$ homozygous mutation in our population (Figure 2).

An indirect consequence of the polymorphism on enzyme function was observed in the wild-type population, in which individuals with high levels of liver enzymes showed relatively normal liver function parameters, including total and indirect bilirubin (Table 2). One member of this population showed some of the highest enzyme levels (142 U/L ALT, 100 U/L AST, and 70 U/L GGT) as well as very low total and indirect bilirubin levels (0.7 and 0.5 mg/dL, respectively), thus reflecting normal functioning of the UGT1A1 enzyme and presumably active HBV (3200 U/L HbsAg) (Brunetto et al., 2010); the AST level regarding this patient is illustrated by a red dot in Figure 1.

In conclusion, our results revealed an association between a *UGT1A1* genotype polymorphism and increased levels of AST. AST catalyzes the transfer of the amino group from the amino acid aspartate to α-ketoglutarate (a keto acid). This process occurs in various tissues, but maximum AST concentrations are observed in the liver.

In diseases that cause liver necrosis, AST is released into the bloodstream where its concentration increases. An increase in serum AST can therefore be found under the following conditions: acute hepatitis (infectious, toxic), chronic hepatitis, necrosis caused by hepatic tumors, and liver metastases.

The large number of subjects who were HBV-positive carriers of heterozygosis or homozygosis for the *UGT1A1*28* $(TA)_7$ polymorphism suggests that these individuals may be more susceptible to cancer and should follow a regime of prevention, adopt a healthy lifestyle (no alcohol or cigarettes), and primarily use drugs for HBV infection that do not affect enterohepatic circulation.

Zehender et al. (2012) showed that genotype D2 entered the Albanian population in the late 1960s and that the effective number of infections increased until the mid-1990s, when it reached a plateau that persists today. The authors suggest that political and socioeconomic factors played an important role in the rapid spread of HBV infection in Albania and highlight that despite a recent decrease in the prevalence of HBsAg in the general population, HBV infection is still highly endemic in the country.

A vaccine for HBV was introduced experimentally in Albania in 1993 and has been available since 1996. A substantial decrease in HBsAg-positive cases is therefore expected in the population aged 18-21 years because of vaccination. Vaccination and a prevention campaign by voluntary associations, as well as centralized quality control of all blood at the national transfusion center in Tirana, where the equipment ensures a high level of control, are changing the outcome of what was considered to be a major health emergency in Albania until recently.

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