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Short communication

POLYMORPHISMS OF THE URIDINE-DIPHOSPHOGLUCURONOSYLTRANSFERASE 1A1 GENE AND CORONARY ARTERY DISEASE

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Abstract: Bilirubin, an antioxidant in the blood, plays a role in protection from atherosclerosis. The level of bilirubin is highly correlated to the incidence of coronary artery disease (CAD). Unconjugated bilirubin is conjugated with glucuronic acid through the reaction of uridine 5'-diphosphate-glucuronosyl transferase 1A1 (UGT1A1). The interactions of CAD and the variations in the coding regions of the UGT1A1 gene have never been evaluated. The purpose of this study was to analyze the influence of the UGT1A1 variant on the incidence of CAD. There were 135 participants in this study: 61 in the experimental group, who had CAD, and 74 in the control group, who did not have CAD. The blood samples from all 135 participants were collected and assayed to clarify the relationship between bilirubin and CAD. The assay of the polymerase chain reaction and the sequence of the UGT1A1 gene were examined to find the gene's polymorphisms. The bilirubin levels for the participants in the control group were significantly higher than for the patients in the CAD group. Although the concentration of bilirubin in the UGT1A1 variant was higher than the wild type for the patients in the CAD group, there was no significant difference in the polymorphism of UGT1A1 between the patients in the CAD group and the participants in the control group.

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Abbreviations used: CAD–coronary artery disease; PCR- polymerase chain reaction; UGT1A1 –uridine 5'-diphosphate-glucuronosyl transferase 1A1

Key words: Atherosclerosis, Coronary artery disease, UGT1A1, Bilirubin, Antioxidant

INTRODUCTION

For many years, bilirubin was considered a waste metabolite from the degradation of the heme. However, recent research has shown that bilirubin is a potent antioxidant and a scavenger of peroxyl radicals [1, 2]. It was reported that bilirubin inhibits complement-dependent inflammation *in vitro* [3]. Several clinical epidemiological studies demonstrated the reverse relationship between the serum bilirubin level and coronary artery disease [4-9]. It was observed that decreased levels of bilirubin were only associated with coronary artery disease (CAD) in men [10, 11].

Bilirubin is converted to bilirubin glucuronide in the liver and excreted into the bile. The hepatic enzyme that catalyzes the glucuronidation of bilirubin is named bilirubin UDP-glucuronosyltransferase (UGT1A1) [12]. Human UGT1A1 gene cDNA was cloned in 1991, and the entire structure of the gene has since been identified [13]. It was reported that only UGT1A1 encoded the enzyme for bilirubin glucuronidation [14]. In previous studies using a group of Taiwanese subjects, it was found that there were five main variation sites of the UGT1A1 gene (Tab. 1) [15]. Recent studies showed another important polymorphism, T-3279G, upstream of UGT1A1. It is located on the phenobarbital response enhancer module (gtPBREM). The incidence of T-3279G and the association between T-3279G and CAD will be analyzed in future studies. There was a racial variability in the polymorphisms of the UGT1A1 gene. Asians have a lower frequency of the A $(TA)_7TA$ allele than Caucasians [16-18]. However,

Tab. 1. The variation sites of the UGT1A1 gene and the relevant amino acid substitutions.

Variation sites	Variation region	Nucleotide change	Amino acid change
$(TA)_6TAA/(TA)_7TAA$	Promoter	None	None
Nucleotide 211	Exon 1	G → A	G → R
Nucleotide 686	Exon 1	C → A	P → Q
Nucleotide 1041	Exon 4	C → T	P → L
Nucleotide 1456	Exon 5	T → G	Y → D

the rate of variation in the coding regions of the gene is comparatively high in Asians [15, 20]. Since a mutation in the UGT1A1 gene could lead to a complete or partial inactivation of the enzyme, individuals with such a mutation would display a decreased excretion of bilirubin and therefore an increased level of bilirubin in the blood [12, 21-23]. Based on the antioxidant potential and the anti-atherogenic properties of bilirubin, it is expected that mutations in UGT1A1

could act with a protective role in CAD. The interactions of CAD and the variations in the coding areas of the UGT1A1 gene had never been evaluated. Bearing in mind that a discrepancy in the genetic basis of UGT1A1 was found between different ethnic groups, this study was conducted to investigate whether there is a difference in the polymorphism of the UGT1A1 gene between people who had and did not have CAD in Taiwan. The relationship between the UGT1A1 genotypes and the bilirubin concentration was also examined.

MATERIALS AND METHODS

The participants in this study were from Chi-Mei Hospital, Tainan, Taiwan. Due to the previous finding of the restriction to gender, only male participants were enrolled. The participants were divided into two groups. The first group was the CAD group, which consisted of 61 patients who had been referred to the Department of Cardiology and diagnosed as having CAD according to the following criteria. For the diagnosis of CAD, a sentinel event or diagnostic study was required. CAD subjects were required to have acute coronary syndrome, coronary catheterization demonstrating significant disease (at least a 50% stenosis in one major coronary artery). Subjects with less than 50% coronary stenosis were excluded from both the CAD group and the control group. The control group consisted of 74 participants who tested healthy in the physical examination, the electrocardiogram and the laboratory examination, including the white blood cell count and cardiac enzyme assays. The CAD group and the control group were entered into this study excluding co-morbidities such as underlying liver disease, elevated liver function tests or hematological disorders.
The consent forms for the participants for this study were obtained before the study began and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.
Blood samples were collected from all the participants after a 12 h fast and prior to the other tests. These serum samples were stored at -80ºC until the analysis of the bilirubin level, which was done with a Diazotized sulfanilic acid reagent with blank correction (Wako Pure Chemical Industries, Osaka, Japan). The data of the lipid profile was also collected.
The genomic DNA was isolated from the whole blood using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). The promoter region containing the (TA)n repeat and the areas of exons 1, 4 and 5 with the variation sites were amplified by polymerase chain reaction (PCR). The PCR was conducted with a volume of 50 µl containing 100-200 ng of genomic DNA, 10 x PCR buffer, 50 ng of each primer, 100 µM of each dNTP, 1.5 mM of $MgCl_2$, and 1 U of *Taq* polymerase. The reaction was performed on a DNA thermal cycler (Perkin Elmer Cetus, Emeryville, CA, USA), followed by three cycles of denaturation at 94ºC for 80 s, annealing at 55ºC for 60 s, and extension at 72ºC for 110 s; seven cycles at 94ºC for 60 s, 55ºC for 60 s, and 72ºC for

110 s; 30 cycles at 94ºC for 50 s, 55ºC for 90 s, and 72ºC for 90 s; and a final extension at 72ºC for 10 min. The oligonucleotide primers used for PCR are presented in Tab. 2.

Tab. 2. The primers used for the PCR of UGT1A1.

	Primers	
Region	Name	Sequence (5'-3')
Promoter	M13-UPF1	M13-GAGTATGAAATTCCAGCCAAG
	UPR1-T7	ACTCTTTCACATCCTCCCTT-T7
Exon 1	M13-U1F1	M13-AGATACTGTTGATCCCAGTG
	U1R1-T7	GCACGTAGGAGAATGGGTTG-T7
	M13-U1F3	M13-CAGCTTTGATGTCATGCTGA
	U1R2-T7	CGATCCAAAGTAATACATCTG-T7
Exon 2	M13-U2F1	M13-ATTCTGTAAGCAGGAACCCT
	U2R1-T7	TAATAGTTGGGAAGTGGCAG-T7
Exon 3	M13-U3F1	M13-ATAGTTCTGCATCCACTTGTT
	U3R1-T7	TGTTACTCACATGCCCTTGC-T7
Exon 4	M13-U4F1	M13-GGCTTAAGCACAGCTATTCT
	U4R1-T7	CATGAATGCCATGACCAAAGT-T7
Exon 5	M13-U5F1	M13-GCCAACATATCCTACATTGC
	U5R1-T7	TGCAGTTAGCCATGCTTGT-T7
None	M13	CACGACGTTGTAAAAACGAC
	T7	TAATACGACTCACTATAGGG

The PCR product was purified via the chaotropic salt method using a commercial DNA purification kit (Quiagen, Ontario, Canada). The purified PCR product was sequenced on a DNA sequencer (4200 DNA analysis system, LI-COR Inc, Lincoln, Nebraska, USA).

The SPSS 11.0 software package (SPSS Inc., Chicago, USA) was applied for the statistical analyses. The non-parametric Mann-Whiney U-Test or t-test for continuous variables and Chi square tests for a dichotomous variable or one way ANOVA for multiple comparisons were used to evaluate if the differences were significant. A two-tailed p value of < 0.05 was considered statistically significant.

RESULTS

The demographics and the clinical characteristics of the study groups are shown in Tab. 3. We also found that there were no significant differences in terms of smoking and hypertension between the control and CAD groups. The levels of bilirubin for the control group were significantly higher than those for the CAD group (0.98 ± 0.30 vs. 0.51 ± 0.22 mg/dl, $P < 0.001$).

There was no significant difference in the percentages of the variations in the UGT1A1 gene between the two groups (Tab. 4). We further analyzed the bilirubin levels according to the different UGT1A1 genotypes. In the CAD group, the participants with UGT1A1 gene variation had a higher concentration of bilirubin than those without the variation. However, there was no difference in the concentration of bilirubin for the respondents in the control group. Among all the participants, a significant difference in bilirubin concentration was only observed in the entire UGT1A1 gene variation versus the wild type (Tab. 5).

Tab. 3. The demographics and clinical characteristics of the participants.

	Control (n = 74)	CAD (n = 61)	P value
Mean Age & Range	59.1 (43-83)	62.7 (40-89)	NS
Hypercholesterolemia, n (%)	49 (66)	41 (67)	NS
Hypertension, n (%)	55 (74)	49 (80)	NS
Smokers, n (%)	35 (47)	34 (56)	NS
Bilirubin, mg/dl*	0.93 (0.72-1.14)	0.34 (0.44-0.64)	<0.001

*The data is presented as median values (25%-75%).

Tab. 4. The percentages for the UGT1A1 genotypes for the CAD and the control groups.

	Non-CAD (n = 74)	CAD (n = 61)	P value
Wild type (n)	51.4% (38)	49.1% (30)	0.80
Variation (n)	48.6% (36)	50.1% (31)	
(TA) 6/6 (n)	88.9% (66)	83.6% (51)	0.34
(TA) 6/7 (n)	11.1% (8)	16.4% (10)	
211 G/G (n)	67.6% (50)	70.5% (43)	0.513
211 G/A (n)	29.7% (22)	26.2% (18)	
211 A/A (n)	2.7% (2)		
686 C/C (n)	97.1% (72)	95.1% (58)	0.82
686 C/A (n)	2.9% (2)	4.9% (3)	
1091 C/C (n)	95.0% (70)	100% (61)	0.18
1091 T C/T (n)	5.0% (4)	0% (0)	
1456 T/T (n)	97.2% (72)	98.3% (60)	0.86
1456 T/C (n)	2.8% (2)	1.7% (1)	

Tab. 5. The bilirubin concentrations (mg/dl) in the different UGT1A1 genotypes.

	Non-CAD (n = 74)	CAD (n = 61)	All (n = 135)
Wild type	0.89 ± 0.24	0.37 ± 0.18	0.57 ± 0.32
Variation	1.00 ± 0.38	0.58 ± 0.17	0.74 ± 0.33
P	0.232	< 0.001	0.014
Promoter (TA)			
6/6	0.95 ± 0.32	0.46 ± 0.20	0.65 ± 0.32
6/7	0.91 ± 0.25	0.62 ± 0.19	0.70 ± 0.24
P	0.94	0.023	0.29
211 G →A			
G/G	0.89 ± 0.23	0.45 ± 0.21	0.60 ± 0.30
G/A	1.07 ± 0.44	0.58 ± 0.18	0.78 ± 0.39
A/A	…	0.64 ± 0.06	0.64 ± 0.06
P	0.123	0.041	0.18

The data is presented as the means and standard deviation.

DISCUSSION

There was sufficient evidence from many studies to suggest that bilirubin acts as an efficient antioxidant and scavenger of peroxyl radicals, and thereby protects lipids and lipoproteins against oxidation [1-3]. Also, many reports supported a reverse association between bilirubin and CAD [4-7]. In this study, the levels of bilirubin for the CAD patients were significantly lower than for the participants in the control group. This result was congruent with the previous findings. It was unclear whether the difference in the levels of bilirubin between participants who had and did not have CAD was caused by gene variation.

Although the concentration of bilirubin for the participants in the control group was significantly higher than for the participants in the CAD group, it was found that the UGT1A1 polymorphisms did not associate with the presence of CAD for the participants in this study. A possible interpretation is that the lower serum concentration of bilirubin in the CAD cases only reflects the consumption of endogenous antioxidant, and is simply a result of increased oxidative stress. It is conceivable that the impact of single nucleotide polymorphism on a complex disease such as CAD may also be influenced by patient selection or lifestyle, or sample size.

The first limitation in this study was the selection bias. The cases of CAD in this study only included patients with stable angina and acute cardiovascular syndromes. Patients with acute myocardial infarction who died before reaching the hospital would never be included in a study. The second limitation was mortality. Several participants in the control group were excluded due to abnormal clinical characteristics. More participants should be included in future

studies. The more participants who can be included, the broader the generalization that can be applied to clarify the relevance of UGT1A1 polymorphisms for CAD.

Heme oxygenase-1 (HO-1), the enzyme catalyzing bilirubin production, is involved in the intracellular antioxidant defense mechanism, and it modulates vascular function [24-26]. The serum bilirubin levels of the control participants were significantly higher than those of the CAD patients in this study, but no difference in the UGT1A1 polymorphisms between the two groups was observed. The influence of HO-1 on bilirubin and CAD in the participants will be further investigated in future studies.

Although it is one of the enzymes involved in the metabolism of bilirubin, UGT1A1 did not seem to influence the levels of bilirubin for the participants in the control group. The impact of the UGT1A1 polymorphisms on bilirubin concentration was only restricted to the CAD group. Statin and aspirin are commonly used for improving the lipid profile and anti-platelet activation for CAD patients. In contrast to the healthy participants in the control group, there is no doubt of the necessity of medication for CAD patients. Of the CAD patients, 57 were taking both statin and aspirin, and 4 had taken statin for the treatment of hypercholesterolemia. It is well known that UGT1A1 is a detoxifying enzyme that also catalyzes glucuronic acid to conjugate many therapeutic drugs [27-29]. An increase in the total bilirubin concentration in participants who were treated with statin drugs has been reported [30, 31]. Grosser *et al.* reported that both aspirin and statin contribute to the induction of HO-1 [32, 33]. For the CAD patients, aspirin and statin may contribute to the significant increase in bilirubin concentration in the UGT1A1 gene variant. In addition to the UGT1A1 gene, other genes or therapeutic drugs for CAD may influence the concentration of bilirubin.

Once the expression of the HO-1 gene increases, the rate of bilirubin production also increases. Since the capability of bilirubin exclusion of the patients with UGT1A1 gene variations is lower, an extensive accumulation of bilirubin is expected. This study demonstrates that bilirubin is an inverse risk factor in CAD. Although the incidence of CAD was not associated with UGT1A1 gene polymorphisms, the bilirubin levels in variants were high among the CAD patients.

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