Association Between the UGT1A1*28 Allele, Bilirubin Levels, and Coronary Heart Disease in the Framingham Heart Study

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Background—Bilirubin is an antioxidant that suppresses lipid oxidation and retards atherosclerosis formation. An inverse association between serum bilirubin and coronary heart disease has been reported. Linkage studies have identified a major locus at the chromosome 2q telomere that affects bilirubin concentrations. A candidate gene in the linkage region encodes hepatic bilirubin uridine diphosphate–glucuronosyltransferase (UGT1A1). The insertion of a TA in the TATAA box of the gene, an allele designated UGT1A1*28, decreases gene transcription. Individuals homozygous for UGT1A1*28 (genotype 7/7) have increased serum bilirubin levels compared with carriers of the 6 allele. To date, no significant association between UGT1A1*28 and cardiovascular disease (CVD) events has been reported. We performed an association study in the Framingham Heart Study population to investigate whether UGT1A1*28 is associated with the risk of CVD events.

Methods and Results—The study population included 1780 unrelated individuals from the Offspring cohort (49% males, mean age 36 years at entry) who had been followed up for 24 years. Individuals with genotype 7/7 had significantly higher bilirubin levels (mean±SD 1.14±0.44 mg/dL) than those with genotypes 6/6 and 6/7 (mean±SD 0.69±0.27 mg/dL, \( P < 0.01 \)). Using the Cox proportional hazards model, we found significant associations between the UGT1A1*28 allele and decreased risk of CVD. Individuals with genotype 7/7 (population frequency of 11%) had approximately one third the risk for CVD and coronary heart disease as carriers of the 6 allele, which resulted in a hazard ratio (95% confidence interval) of 0.36 (0.18 to 0.74) and 0.30 (0.12 to 0.74), respectively.

Conclusions—Homozygote UGT1A1*28 allele carriers with higher serum bilirubin concentrations exhibit a strong association with lower risk of CVD. (Circulation. 2006;114:1476-1481.)

Key Words: cardiovascular diseases ■ genetics ■ genes ■ atherosclerosis ■ enzymes ■ epidemiology ■ survival

Many studies have reported that low serum bilirubin concentrations are associated with an increased risk of coronary heart disease (CHD) events.1–5 The strength of the association was similar to that of smoking, elevated systolic blood pressure, and low levels of high-density lipoprotein (HDL) cholesterol.6,7 In a retrospective case-control study of individuals with early familial CHD onset, higher serum bilirubin concentrations within the normal range were associated with a significant and marked reduction in CHD risk.3 Individuals with Gilbert syndrome who have mildly elevated bilirubin levels were found to have an ischemic heart disease rate of 2% compared with 12.1% in the general population.8 In a prospective study of serum bilirubin and cardiovascular disease (CVD) in the Framingham Offspring Study, higher serum bilirubin concentrations were associated with decreased risk of CVD, CHD, and myocardial infarction (MI). A recent experimental study also suggested that bilirubin might have a potential therapeutic value in vascular proliferative disorders.10

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Oxidized low-density lipoprotein (LDL) may play an important role in the atherosclerotic process. Studies indicate that bilirubin, the metabolic waste product of heme degradation, is an effective antioxidant that efficiently scavenges peroxyl radicals and suppresses the oxidation of lipids and lipoproteins, especially LDL, and thus acts against plaque formation and subsequent atherosclerosis. Bilirubin is a more
effective protector of human ventricular myocytes than several known antioxidants, such as vitamin C and vitamin E analogues.\textsuperscript{11} Bilirubin acts as an antioxidant whether it is unconjugated, conjugated, free, or albumin bound.\textsuperscript{11–13}

Previous linkage studies identified a major locus at the chromosome 2q telomere that affects bilirubin concentrations.\textsuperscript{14,15} These 2 studies revealed a logarithm of the odds score of 3.8 and 3.2, respectively, spanning a region of 22 centimorgans (logarithm of the odds–1 supporting interval). One obvious candidate gene under the linkage peak is the gene that encodes hepatic bilirubin uridine diphosphate–glucuronosyltransferase (UGT1A1), which is the only enzyme that contributes substantially to bilirubin glucuronidation and thus enhances bilirubin elimination. Mutations in this gene have been implicated in the mild hyperbilirubinemic condition, Gilbert’s syndrome,\textsuperscript{16} and a more severe childhood form of hyperbilirubinemia, the Crigler-Najjar syndrome.\textsuperscript{17–19}

A common cause of decreased UGT1A1 activity is the insertion of a TA in the TATAA box in the promoter region of the UGT1A1 gene, designed as UGT1A1*28.\textsuperscript{16} Individuals homozygous for 7 repeats (7/7) have higher levels of serum bilirubin than those who are heterozygotes (6/7) or the wild type of 6 repeats (6/6).\textsuperscript{16,20,21} The allele frequency of this polymorphism among the white population is extraordinarily high, 34% to 40%, and the frequency of individuals homozygous for 7 repeats is 10% to 16%.\textsuperscript{16,22}

To date, only the Rotterdam Study investigated an association of the UGT1A1*28 allele and CHD.\textsuperscript{23} In the Rotterdam Study, neither bilirubin nor UGT1A1*28 was associated with CHD. In the present study, we report the results of associations between the UGT1A1*28 allele, bilirubin levels, and CVD events in the Framingham Offspring Study.

Methods

Study Subjects

The Framingham Heart Study has investigated risk factor determinants of CVD over decades in a general population.\textsuperscript{24} In brief, the Framingham Heart Study began in 1948 with the recruitment of 5209 residents aged 28 to 62 years in approximately two thirds of the households in the town of Framingham, Mass. Participants have undergone biennial examinations since the study began. In 1971, the Framingham Offspring Study\textsuperscript{25} was begun, in part to evaluate the role of genetic components of CVD causes. In total, there were 5124 subjects aged 5 to 70 years at entry, including the children of the original cohort and their spouses. The Offspring cohort has been examined every 4 years (except for the 8-year interval between the first 2 examinations). The Framingham Heart Study consists almost entirely of subjects of European descent from England, Ireland, France, and Italy.

A sample of 1888 unrelated individuals was randomly selected from the Offspring generation from the 3532 subjects attending the sixth examination cycle during 1995 to 1998, and these individuals had blood samples collected for DNA extraction. To be eligible for the unrelated sample, the selected subjects had to be unrelated to other subjects in the sample (ie, 1 person randomly selected from each extended family or biologically unrelated to any other Framingham participant). Additionally, the selection was designed to include equal numbers of men and women (as opposed to 56% women in the entire cohort). These criteria were defined to provide a panel of DNAs that would be suitable for multiple studies and were not specific to any traits. The selected subjects were similar to other Framingham Offspring cohort members with respect to the prevalence of CVD events and risk factors, except for being slightly older (mean age at the sixth examination cycle 60 versus 58 years; \(P=0.0001\)) and more likely to be hypertensive (44% versus 39%; \(P=0.001\)). Among the unrelated subjects available for the present study, 1780 had available genotype information and 49% were males; the age range was 13 to 62 years (mean age 36 years) at entry into the present study.

Phenotype Measurements

The average number of cigarettes smoked per day over the prior year was determined by self-reporting. Laboratory measurements were made on 12-hour fasting venous blood samples that were collected in tubes that contained 0.1% ethylenediaminetetraacetic acid. Lipid determinations were performed at the Framingham Heart Study laboratory, which participated in the Standardization Program of the Centers for Disease Control. Total serum bilirubin was measured during the first examination with the colorimetric method. The clinical and laboratory methods have been detailed elsewhere.\textsuperscript{24}

At each examination, information about the occurrence of interim cardiovascular events was collected with a standardized questionnaire administered by a physician. Any subject with a possible or definite end point was independently interviewed by a second physician. A review panel of 3 physicians made the final determination of cardiovascular end points. A detailed description of the cardiovascular events has been published.\textsuperscript{26} Three cardiovascular events (CHD, CVD, and MI) were analyzed. The CHD category consisted of fatal and nonfatal MI, angina pectoris, or coronary insufficiency. The CVD category consisted of CHD, stroke, transient ischemic attack, intermittent claudication, congestive heart failure, or CHD death. All subjects provided informed consent before each clinic visit, and the examination protocol was approved by the Institutional Review Board at Boston University Medical Center, Boston, Mass.

Genotyping

The UGT1A1 promoter polymorphism was analyzed on the ABI 3130xl sequencing system (Applied Biosystems, Foster City, Calif) in a manner similar to that described recently.\textsuperscript{27} Briefly, polymerase chain reaction was performed with a 5-FAM (carboxyfluorescein)–labeled forward primer (5’-CACGTGACACAGTCAAAAC-3’) and an unlabeled reverse primer (5’-CACAGTATCTTCCACGC-3’). Each 5-μL polymerase chain reaction mixture included 0.25 U of HotStarTaq DNA polymerase (Qiagen, Valencia, Calif), 1× Q-Solution (Qiagen), 1× Taq buffer (Qiagen), 2.5 mmol/L MgCl\(_2\) (Qiagen), 0.25 mmol/L dNTPs (Sigma, St. Louis, Mo), 1 μmol/L of each primer, and 10 ng of DNA. The thermal cycling was performed in 384-well plates (ABgene, Epsom, United Kingdom) on an ABI Prism 7900HT system (Applied Biosystems) under the following conditions: 95°C for 15 minutes followed by 28 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final extension of 94°C for 10 minutes.

For fragment analysis, 1 μL of the polymerase chain reaction product was mixed with 9 μL of HiDi-Formamide (Applied Biosystems) and 0.5 μL of the Genscan 400HD ROX size standard (Applied Biosystems) in 96-well detection plates (ABgene). After a denaturation (95°C for 3 minutes) and cooling step (4°C for 5 minutes) on a TGradient Thermocycler (Biorad, Richmond, Calif), the fragments were analyzed on the ABI 3130xl sequencing system. All pipetting steps were executed by an EVO150 robotic system (Tecan, Zurich, Switzerland).

For data analysis, we applied GeneMapper version 3.7 software (Applied Biosystems). Genotyping was done within the genotyping unit of the Gene Discovery Core Facility at the Innsbruck Medical University, Innsbruck, Austria.

Statistical Analysis

In addition to 6 and 7 repeat alleles, we also found 10 subjects with a 5 repeat allele and 2 with an 8 repeat allele. Because of the low frequencies and experimental studies reporting decreasing promoter activity with an increasing number of repeats,\textsuperscript{23} we combined the 5 repeat with the 6 repeat allele and the 8 repeat with the 7 repeat allele.
Results

Table 1 presents the characteristics of the study population stratified by UGT1A1*28 genotype. Genotype frequencies were 46%, 43%, and 11% for genotypes 6/6, 6/7, and 7/7, respectively. The distribution of the genotypes was in Hardy-Weinberg equilibrium (P=0.82). No significant differences across genotype groups were found in age at entry, sex, HDL cholesterol level, LDL cholesterol level, systolic blood pressure, diastolic blood pressure, or smoking status. Mean serum bilirubin was highest in 7/7 carriers (1.14 ± 0.44, n = 191) and lower in 6/7 (0.75 ± 0.30, n = 769) and 6/6 (0.63 ± 0.23, n = 820) carriers (P < 0.01). The total variation of bilirubin explained by the gene after adjustment for covariates was 18.6% (R² = 27.1%) for the full model with the gene and covariates and 8.5% for the submodel with covariates only. Table 2 displays the relationship between bilirubin and CVD events. Bilirubin levels were significantly lower in subjects with a CVD or CHD event than in individuals free of those complications. This association was of borderline significance for the MI event group.

When we investigated the association of the UGT1A1 polymorphism with each outcome, we observed that outcome rates were very similar and that there were no major differences between the 6/7 and 6/6 genotype groups for all 3 types of events. This implies that a recessive model would best fit the observed data. Therefore, we combined these 2 genotype groups, using the combined group as a reference for comparison with the genotype 7/7 group, and performed post hoc tests. Table 3 represents the cumulative number of outcomes recorded at the end of 24 years of follow-up, and the Figure provides the survival curves stratified by the combined 6/6+6/7 and 7/7 genotypes for the 3 CVD event groups. For CVD and CHD, there were significantly decreased numbers of events in the 7/7 homozygotes compared with those of the combined group. Individuals with genotype 7/7 had approximately one third the risk for CVD and CHD as carriers of the 6 allele, which resulted in a hazard ratio (95% confidence interval) of 0.36 (0.18 to 0.74) and 0.30 (0.12 to 0.74), respectively. A similar result was seen for MI, but this did not reach statistical significance (hazard ratio 0.52, 95% confidence interval 0.19 to 1.43). We also considered smoking as an effect modifier and performed a stratified analysis of smoking status instead of using it as a covariate; no differ-

### Table 1. General Characteristics of the Study Population Stratified by UGT1A1*28 Genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>6/6 (820)</th>
<th>6/7 (769)</th>
<th>7/7 (191)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total typed, n (%)</td>
<td>820 (46.0)</td>
<td>769 (43.2)</td>
<td>191 (10.8)</td>
</tr>
<tr>
<td>Age at entry, y</td>
<td>36.1 (9.2)</td>
<td>35.7 (9.7)</td>
<td>36.5 (9.5)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>50.4 (15.1)</td>
<td>51.1 (13.9)</td>
<td>50.4 (14.7)</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>123.8 (33.8)</td>
<td>123.8 (35.0)</td>
<td>123.0 (31.3)</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>121.0 (15.0)</td>
<td>120.3 (15.4)</td>
<td>120.9 (13.0)</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>78.4 (10.7)</td>
<td>77.5 (10.7)</td>
<td>78.4 (9.9)</td>
</tr>
<tr>
<td>Smoking, n/d</td>
<td>13.5 (14.8)</td>
<td>13.3 (14.2)</td>
<td>15.6 (18.0)</td>
</tr>
<tr>
<td>Bilirubin, mg/dL*</td>
<td>0.63 (0.23)</td>
<td>0.75 (0.30)</td>
<td>1.14 (0.44)</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; DBP, diastolic blood pressure. Values are mean (SD) except for total typed. *P < 0.01

### Table 2. Bilirubin in Cases and Noncases* of CVD Event Groups

<table>
<thead>
<tr>
<th>Event Groups</th>
<th>Cases</th>
<th>Noncases</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD</td>
<td>156</td>
<td>1583</td>
<td>0.001</td>
</tr>
<tr>
<td>CHD</td>
<td>117</td>
<td>1583</td>
<td>0.002</td>
</tr>
<tr>
<td>MI</td>
<td>59</td>
<td>1583</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Bilirubin values are mean (SD). *Those free of CVD were used as the comparison group for all events.

### Table 3. Cumulative CVD Events and Hazard Ratios (95% Confidence Interval) of UGT1A1*28 Genotype and Bilirubin Levels at 24 Years of Follow-Up

<table>
<thead>
<tr>
<th>Genotype 6/6 (820)</th>
<th>Genotype 6/7 (769)</th>
<th>Genotype 7/7 (191)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD cases</td>
<td>79 (4.24)</td>
<td>69 (3.93)</td>
</tr>
<tr>
<td>CHD cases</td>
<td>60 (3.18)</td>
<td>52 (2.92)</td>
</tr>
<tr>
<td>MI cases</td>
<td>26 (1.34)</td>
<td>29 (1.60)</td>
</tr>
<tr>
<td>Hazard Ratio (95% Confidence Interval)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For 7/7 vs 6/6+6/7 Bilirubin</td>
<td>0.36 (0.18–0.74), P = 0.005</td>
<td>0.90 (0.84–0.96), P = 0.003</td>
</tr>
<tr>
<td>For 0.1 mg/dL Bilirubin</td>
<td>0.30 (0.12–0.74), P = 0.009</td>
<td>0.87 (0.81–0.95), P = 0.001</td>
</tr>
<tr>
<td>For 7/7 vs 6/6+6/7, Adjusted for Bilirubin</td>
<td>0.52 (0.19–1.43), P = 0.202</td>
<td>0.87 (0.78–0.97), P = 0.013</td>
</tr>
</tbody>
</table>

Mean serum bilirubin was highest in 7/7 carriers (1.14 ± 0.44, n = 191) and lower in 6/7 (0.75 ± 0.30, n = 769) and 6/6 (0.63 ± 0.23, n = 820) carriers (P < 0.01). The total variation of bilirubin explained by the gene after adjustment for covariates was 18.6% (R² = 27.1%) for the full model with the gene and covariates and 8.5% for the submodel with covariates only.
ences in associations between the polymorphism and CVD events were observed for the 3 event groups (data not shown), however, and these associations were similar to those of the unstratified results.

As a next step, we investigated the association of bilirubin serum levels with each outcome: A 0.1-unit increase in bilirubin levels decreased CVD, CHD, and MI risk by 10%, 13%, and 13%, respectively, when the genotype was not included in the model. After we included both the UGT1A1 polymorphism and bilirubin levels in the Cox regression model, the UGT1A1 effect disappeared (Table 3). Because hemoglobin is a breakdown product of heme, we also examined a model with hemoglobin instead of bilirubin; however, hemoglobin did not change the association of the UGT1A1 polymorphism with the outcomes and therefore is unlikely to be a potential confounder (data not shown).

Discussion

In the present study with 24 years of follow-up in 1780 participants of the Framingham Offspring Study, we found for the first time a significantly decreased risk of CVD and CHD for subjects with the UGT1A1*28 7/7 genotype. Individuals with this genotype had approximately one third the risk for CVD and CHD of those with genotypes 6/6 or 6/7. The polymorphism was not statistically significantly associated with MI, although there was a similar trend. One possible explanation is inadequate power owing to the low number of incident MIs. We confirmed previous findings that low serum bilirubin levels were associated with higher risk of CVD, CHD, and MI. For each 0.1-unit increase in bilirubin levels, CVD, CHD, and MI risks decreased 10%, 13%, and 13%, respectively. Allele 7 was significantly associated with higher serum bilirubin, and the gene explained a large proportion of the variation in bilirubin levels. In Cox models that added serum bilirubin, the association with the UGT1A1 polymorphism was no longer significant. This result suggests that the bilirubin variability that results from UGT1A1*28 variation may be an intermediate phenotype for CVD events.

Many studies have reported an association between UGT1A1*28 and serum bilirubin levels and an association between bilirubin levels and CVD events. Only the Rotterdam Study to date has investigated the association between UGT1A1*28 and CVD events, and that study found that the association was not statistically significant.23 Moreover, low serum bilirubin concentrations were not found to be associated with an increased risk of CHD. The authors acknowledged that the protective effect might have been missed because of a lack of power to detect such an effect. In addition to this, there are several other differences in the 2 study cohorts that might explain the differences in the results. First, the participants in the Rotterdam Study were elderly subjects, with a mean age of almost 70 years at baseline. This contrasts markedly from the Framingham Heart Study, in which the participant age was on average 36 years at baseline. Genetic factors for CVD may be easier to detect in a young group with a long follow-up than in an elderly group with short follow-up, in which many other factors, including environmental factors, might have contributed to a high baseline incidence of atherosclerosis. Second, a survival bias in the Rotterdam Study due to the older ages at baseline is possible, because many individuals do not survive a CVD event and thus...
may not be available for inclusion into the study. A survival bias is also possible but is likely minimal in the Framingham Study because of the young age at study entry. Third, for a variety of reasons, only approximately half of the cases in the Rotterdam Study (185/347) were included in the final analysis, and bilirubin levels were only available in 114 cases and 162 controls. We cannot exclude the possibility of an inadvertent selection bias that resulted from missing individuals. Finally, whether population substructure influenced the results of the Rotterdam Study was not investigated. A recent study reported no evidence of major population substructure in the Framingham Heart Study.

Allelic association can be explained by linkage disequilibrium with a nearby susceptibility locus, population substructure, or direct biological action of the polymorphism. Functional studies on that polymorphism support a causative role in the development of atherosclerosis; Bosma et al. demonstrated that gene expression in the presence of 7 repeats was 3 to 5 times lower than that of the wild type of 6 repeats. In a study investigating the correlation between this polymorphism and in vitro human liver UGT1A1 enzyme activity, the enzyme activity of subjects with the 6/6 genotype was significantly higher than the activity of subjects with 6/7 and 7/7 genotypes. Therefore, the insertion of the TA in the promoter region of UGT1A1 is a functional mutation itself and not in linkage disequilibrium with a susceptibility locus. Because UGT1A1 is a rate-limited enzyme for bilirubin excretion, serum bilirubin concentrations may mainly be determined by excretion.

Two segregation studies indicated that higher serum bilirubin segregated as a major gene trait and may protect against CHD. Given the estimated allele frequency and its effect on serum bilirubin, the UGT1A1*28 allele is a likely candidate for the reported trait. If the population has a strong protective effect against CHD because of UGT1A1*28, this would have significant population implications in future CVD prevention.

In summary, the present study demonstrates a significant association between UGT1A1*28 and cardiovascular events. Further studies are warranted to confirm this association.

Sources of Funding

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Disclosures

None.

References


Blood lipids and lipoprotein oxidation may play an important role in the atherosclerotic process. Bilirubin is an effective antioxidant that efficiently suppresses oxidation of lipids and lipoproteins. Low serum bilirubin concentrations are associated with an increased risk of cardiovascular disease events. Linkage and association studies have suggested that the gene encoding hepatic bilirubin uridine diphosphate–glucuronosyltransferase (UGT1A1), which is the only enzyme that contributes substantially to bilirubin glucuronidation and thus enhances bilirubin elimination, may be one of the major genes in the control of the variation of serum bilirubin levels. A common cause of decreased UGT1A1 activity is the insertion of a TA in the TATAA box in the promoter region of the UGT1A1 gene, designed as UGT1A1*28. Individuals homozygous for 7 repeats (7/7; population frequency of 10% to 16%) have higher levels of serum bilirubin than carriers of the wild type of 6 repeats (6/6). In 1780 participants of the Framingham Heart Study followed up for 24 years, individuals with genotype 7/7 had significantly higher bilirubin levels than carriers of the 6 allele and had approximately one third the risk for cardiovascular disease and coronary heart disease as carriers of the 6 allele. The present study suggests that this polymorphism may have a substantial impact on the development of cardiovascular disease and thus may be an important target for therapeutic intervention.
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