

men classified as unlikely to be, uncertain to be, or likely to be vitamin B₁₂ deficient (Table 1A). HoloTC was significantly ($P < 0.01$) lower in the group of vegan men unlikely to have vitamin B₁₂ deficiency compared with omnivores, whereas this was not the case for B12 ($P = 0.30$; Table 1A).

Finally we examined whether holoTC might replace combined testing with B12, MMA, and tHcy. In this analysis, individuals were classified as likely to have vitamin B₁₂ deficiency if at least two of three tests were outside the cutoff limits (B12 < 120 ng/L, MMA > 0.75 μ mol/L, tHcy > 15 μ mol/L; $n = 56$) or not ($n = 139$; all others). The diagnostic accuracy of holoTC was then assessed by ROC curve analysis (Fig. 1B). The areas under the ROC curves were 0.91 (95% CI, 0.87–0.95) for all individuals, 0.88 (95% CI, 0.82–0.95) for vegan men recruited from the EPIC study, and 0.92 (95% CI, 0.85–0.99) for the men recruited from the London Vegan Society.

We also compared holoTC and the other three markers in omnivores and in vegan men classified as unlikely to be, uncertain to be, or likely to be vitamin B₁₂ deficient based on test results for B12, MMA, and tHcy (Table 1B). HoloTC was significantly lower ($P = 0.017$) in the vegan men who were unlikely to be vitamin B₁₂ deficient compared with the omnivores, whereas no significant difference was observed for the other three measures: B12 ($P = 0.49$), MMA ($P = 0.37$), and tHcy ($P = 0.47$).

Each of the laboratory tests used for diagnosis of vitamin B₁₂ deficiency has its weaknesses. The metabolites MMA and tHcy are influenced by kidney function (10, 11), and tHcy also depends on the folate status of the patient. B12 is influenced by the concentration of the binding proteins of vitamin B₁₂ and is increased in patients with myeloproliferative diseases (12).

Our data are consistent with an early decrease of holoTC in vitamin B₁₂ deficiency in vegans and further suggest that holoTC might replace combined testing with B12, MMA, and tHcy in this population group. If holoTC is used as the primary screening test, we suggest no further testing for patients with holoTC > 50 pmol/L (unlikely to suffer from vitamin B₁₂ deficiency) and holoTC < 25 pmol/L (likely to suffer from vitamin B₁₂ deficiency). For patients with holoTC between 25 and 50 pmol/L, we would suggest further testing with one of the metabolic markers.

Further studies are needed to evaluate the validity of holoTC in other patient groups, especially those with conditions likely to influence markers of vitamin B₁₂ deficiency. Measurement of holoTC may be of particular value in identifying vitamin B₁₂ deficiency in patients with kidney malfunction, in whom the metabolites may show falsely increased values (10, 11), and in patients with myeloproliferative diseases, in whom the concentration of B12 may be falsely increased (12). We conclude that holoTC is a promising indicator of vitamin B₁₂ deficiency.

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Haplotype Structure of the UDP-Glucuronosyltransferase 1A1 (UGT1A1) Gene and Its Relationship to Serum Total Bilirubin Concentration in a Male Korean Population, Chang-Seok Ki,¹ Kyung-A Lee,² Soo-Youn Lee,¹ Hee-jin Kim,¹ Sang Sun Cho,³ Jun-Hee Park,³ Seunghee Cho,³ Kwang Min Sohn,³ and Jong-Won Kim^{1*} (¹ Department of Laboratory Medicine, Sungkyunkwan University School of Medicine, Samsung Medical Center, Ilwon-Dong, Kangnam-Gu, Seoul 135-710, Korea; ² Department of Laboratory Medicine, College of Medicine, Korea University, Anam-Dong, Seongbuk-Gu, Seoul, Korea; ³ Clinical Research Center, Samsung Biomedical Research Institute, Ilwon-Dong, Kangnam-Gu, Seoul, Korea; * author for correspondence: fax 82-2-3410-2719; e-mail jwonk@smc.samsung.co.kr)

UDP-glucuronosyltransferase 1A1 (UGT1A1) is the key enzyme for bilirubin conjugation. Defects in this enzyme can cause a nonhemolytic unconjugated hyperbilirubinemia, such as Crigler-Najjar syndrome type 1 (CN1)

and 2 (CN2) and Gilbert syndrome (GS). In 1991, the cDNA of the human *UGT1A1* gene was cloned, and this led to the identification of genetic defects in patients with CN1, CN2, and GS (1–3). It was shown that homozygous or compound heterozygous mutations of the *UGT1A1* gene can lead to these inheritable unconjugated hyperbilirubinemias, and >30 variants have been identified (4, 5).

In GS, a TATAA box variant [A(TA)₆TAA>A(TA)₇TAA] in the promoter region of the *UGT1A1* gene has been reported in Caucasian populations, and several polymorphisms in the coding region, including 211G>A (G71R), have been reported to have similar associations with GS in Japanese populations (6–8). Recently, Sugatani et al. (9) identified a T-to-G substitution in the phenobarbital-responsive enhancer module 3279 bp upstream from the *UGT1A1* gene. They suggested that the –3279T>G polymorphism could be another risk factor for the development of mild hyperbilirubinemia.

Presumably, different combinations of the polymorphisms (haplotypes) in the *UGT1A1* gene associated with GS or mild hyperbilirubinemia might produce a variety of serum total bilirubin (T-Bil) concentrations. Because these polymorphisms in the *UGT1A1* gene lie in a relatively small region (Fig. 1A), a certain extent of linkage disequilibrium (LD) among these polymorphisms is expected. Therefore, haplotype analysis is more reasonable than association analysis using any single polymorphism to reveal the genetic background of an increased serum T-Bil concentration. We analyzed the haplotype structure of the *UGT1A1* gene and investigated its relationship to the serum T-Bil concentration in healthy Korean males.

The study participants were 324 healthy Korean males [mean (SD) age, 49.8 (5.4) years] randomly selected from the registry for routine health checks at Samsung Medical Center in Seoul, Korea. We restricted the study participants to males because the prevalence of unconjugated hyperbilirubinemia is higher in men than in women because of the lower rate of daily bilirubin production or other unidentified factors in females (10, 11) and thus the genetic influence on the bilirubin concentration might be

more evident in men. None had a history of hepatic or hematologic disorders such as anemia, excessive alcohol intake, or chronic use of medications or narcotics. Informed consent was obtained from all participants, and the Institutional Review Board of the Samsung Medical Center approved the study protocol. Serum samples were collected from each participant in the morning after overnight fasting. The serum T-Bil concentration was measured by the diazo method (Daiichi Pure Chemicals) with the Hitachi 747 system (Hitachi) at least twice with a >1-month interval to provide a mean value for each participant. The mean values were used in all statistical analyses.

Two promoter regions containing the –3279T>G polymorphism and the TATAA box, and a part of exon 1 containing the 211G>A polymorphism of the *UGT1A1* gene were amplified and sequenced with use of appropriate primers, as given in the Data Supplement that accompanies the online version of this Technical Brief (available at <http://www.clinchem.org/content/vol49/issue12/>). The differences in the serum T-Bil concentration among the subgroups according to the *UGT1A1* alleles or haplotypes were tested by ANOVA. The influence of smoking status, polymorphisms, and haplotypes of the *UGT1A1* gene on serum T-Bil was also estimated by the GLM procedure incorporated in the SAS System for Windows (SAS Institute Inc.). The Hardy–Weinberg equilibrium was tested with Arlequin software (12). Multisite haplotypes and their frequencies were estimated with use of SAS Genetics software (SAS Institute Inc.), and the assignment of haplotypes to each individual was performed with the PHASE program (13). Pairwise LD was estimated as D , D' , and r^2 (14). These LD coefficients were calculated with use of the SAS Genetics software.

The mean (SD) serum T-Bil concentration in the total group of participants was 16.3 (5.9) $\mu\text{mol/L}$ [0.95 (0.34) mg/dL]. When we investigated the association of smoking with serum T-Bil concentrations, we observed a significant difference among groups by smoking status ($P = 0.0058$, ANOVA). Current smokers had lower serum T-Bil concentrations [14.3 (5.3) $\mu\text{mol/L}$; $n = 71$] compared with

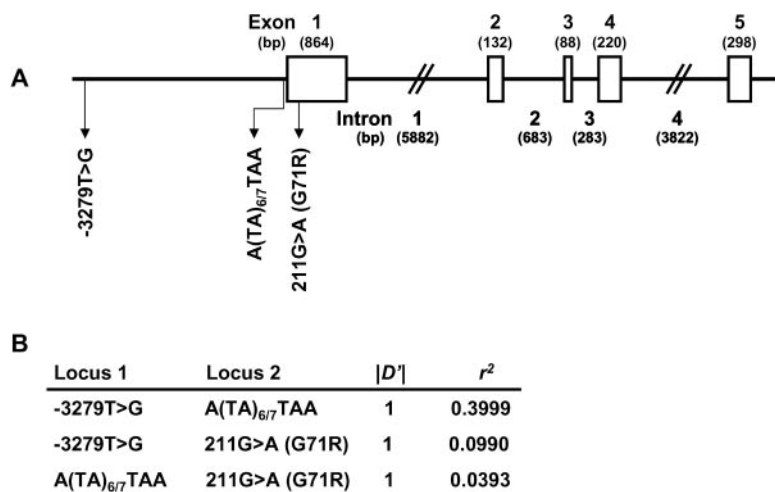


Fig. 1. Schematic presentation of the *UGT1A1* gene (A), and LD coefficients (B).

(A), exact sizes of exons (open boxes) and introns are based on the Ensembl transcript ENST00000330771 (<http://www.ensembl.org>). Locations of three common polymorphisms are indicated by arrows. (B), Lewontin's coefficient (D') and correlation coefficient (r^2) between each pair of the three polymorphisms.

never smokers [17.9 (6.6) $\mu\text{mol/L}$; $n = 45$], and former smokers had serum T-Bil concentrations [16.8 (5.8) $\mu\text{mol/L}$; $n = 155$] between those of current smokers and never smokers. Smoking status was estimated to explain $\sim 3.8\%$ of the variation in serum T-Bil concentrations.

The genotype frequencies of all three polymorphisms were in Hardy–Weinberg equilibrium, and the frequencies of the -3279G , $(\text{TA})_7$, and 211A alleles were calculated as 0.267, 0.127, and 0.213, respectively. As shown in the table in the online Data Supplement, the mean serum T-Bil concentrations in homozygous carriers of the -3279G , $(\text{TA})_7$, or 211A allele were significantly higher than those in heterozygous carriers or homozygous carriers of wild-type alleles ($P < 0.0001$). Among the three polymorphisms, homozygous carriers of $(\text{TA})_7$ showed the highest mean serum T-Bil concentration [33.2 (4.1) $\mu\text{mol/L}$], followed by homozygous carriers of 211A [24.2 (8.3) $\mu\text{mol/L}$] and -3279G [23.7 (6.2) $\mu\text{mol/L}$]. The variabilities in serum T-Bil explained by the $-3279\text{T}>\text{G}$, $(\text{TA})_{6/7}$, and 211G>A polymorphisms were $\sim 15.1\%$ ($P < 0.0001$), 28.1% ($P < 0.0001$), and 12.9% ($P < 0.0001$), respectively.

A likelihood ratio test using Arlequin software (12) detected significant pairwise LD with D' values of 1.0 between all pairs of the three polymorphisms ($P < 0.0001$; Fig. 1B). The highest correlation was observed between $-3279\text{T}>\text{G}$ and $(\text{TA})_{6/7}$ polymorphisms ($r^2 = 0.3999$; Fig. 1B). Multisite haplotype inference revealed that the participants had four of eight possible haplotypes: 3279T-(TA)₆-211G (ht1), 3279T-(TA)₆-211A (ht2), 3279G-(TA)₆-211G (ht3), and 3279G-(TA)₇-211G (ht4). ht1 was the most common with an estimated frequency of 0.5201 [95% confidence interval (CI), 0.4816–0.5586], followed by ht2 (0.2129; 95% CI, 0.1814–0.2445), ht3 (0.1404; 95% CI, 0.1137–0.1672), and ht4 (0.1265; 95% CI, 0.1009–0.1521).

When the participants were stratified into 10 groups according to their *UGT1A1* haplotypes, we observed significant differences in the mean serum T-Bil concentration among the groups ($P = 0.0001$; Table 1). The lowest concentration was observed in group A [ht1/ht1; 13.1 (3.6) $\mu\text{mol/L}$], whereas the highest concentration was observed in group J [ht4/ht4; 33.2 (4.1) $\mu\text{mol/L}$]. Homozygous as well as compound heterozygous carriers of the variant haplotypes (ht2, ht3, or ht4) had increased serum T-Bil concentrations compared with homozygous or heterozygous carriers of the wild haplotype (ht1). Approximately 61.4% and 53.5% of the variability in serum T-Bil concentrations could be explained by the *UGT1A1* haplotype with or without consideration of smoking status, respectively.

Multisite haplotype inference revealed that our study population had only four of eight possible haplotypes. The four missing haplotypes, 3279T-(TA)₇-211G, 3279T-(TA)₇-211A, 3279G-(TA)₆-211A, and 3279G-(TA)₇-211A, suggest that -3279T plus $(\text{TA})_7$ and $(\text{TA})_7$ plus 211A alleles never exist on the same chromosome, at least in a Korean population. From the above results, we also suggest that a transition of G to A at nucleotide 211 in exon 1 of the *UGT1A1* gene would form the ht2 haplotype,

Table 1. Haplotype groups and T-Bil concentrations based on different combinations of three polymorphisms of the *UGT1A1* gene in 324 healthy Korean males.

Group	Haplotype ^a	n (%)	Mean (SD) T-Bil ^b	
			$\mu\text{mol/L}$	mg/dL
A	ht1/ht1	90 (27.8)	13.1 (3.6)	0.76 (0.21)
B	ht1/ht2	66 (20.4)	15.5 (3.1)	0.91 (0.18)
C	ht2/ht2	18 (5.6)	23.7 (6.2)	1.38 (0.36)
D	ht1/ht3	54 (16.7)	13.5 (2.8)	0.79 (0.16)
E	ht2/ht3	17 (5.2)	16.9 (3.4)	0.99 (0.20)
F	ht1/ht4	37 (11.4)	16.3 (4.5)	0.95 (0.26)
G	ht2/ht4	19 (5.9)	24.6 (5.1)	1.44 (0.30)
H	ht3/ht3	4 (1.2)	18.8 (1.7)	1.10 (0.10)
I	ht3/ht4	12 (3.7)	20.7 (7.3)	1.21 (0.43)
J	ht4/ht4	7 (2.2)	33.2 (4.1)	1.94 (0.24)

^a ht1, 3279T-(TA)₆-211G; ht2, 3279T-(TA)₆-211A; ht3, 3279G-(TA)₆-211G; ht4, 3279G-(TA)₇-211G.
^b There were significant differences in concentrations among the genotype groups ($P < 0.0001$, ANOVA).

whereas a transversion of T to G at nucleotide -3279 in the promoter region of the *UGT1A1* gene would form the ht3 haplotype. In addition, ht4 could be the result of an introduction of an extra (TA) repeat to the ht3.

Disclosure of the molecular genetic basis of reduced expression of *UGT1A1* is important not only for understanding the molecular pathophysiology of increased serum T-Bil concentrations in GS but also for predicting severe toxicity by irinotecan in cancer patients (15, 16). In addition, increased serum T-Bil concentrations have been associated with a low risk of coronary artery disease (17, 18). To reveal the genetic background of an increased serum T-Bil concentration, haplotype analysis is more reasonable than association analysis using any single polymorphism because of tight LD among polymorphisms in the *UGT1A1* gene.

In summary, we demonstrated that there is complete LD among three common polymorphisms of the *UGT1A1* gene in a male Korean population. In addition, we could unequivocally construct haplotypes for the *UGT1A1* gene, which were revealed to be significantly associated with the serum T-Bil concentration. However, there are two major limitations in the present study. One is that the study population comprised only Korean males and thus the results cannot be freely applied to other populations, and the other is that only three common polymorphisms were included in the analysis. Nevertheless, to the best of our knowledge, this is one of the first studies to analyze the haplotype structure of the *UGT1A1* gene including both promoter and coding regions and to assess the haplotype–phenotype correlation between the *UGT1A1* gene and the serum T-Bil concentration.

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Recombinant Human Intrinsic Factor Expressed in Plants Is Suitable for Use in Measurement of Vitamin B₁₂, Mustafa Vakur Bor,^{1*} Sergey N. Fedosov,² Niels B. Laursen,^{2,3} and Ebba Nexø¹ (¹Department of Clinical Biochemistry, AKH; Aarhus University Hospital, DK-8000 Aarhus C, Denmark; ²The Protein Laboratory, Aarhus University, DK-8000 Aarhus C, Denmark; ³Cobento Biotech A/S, DK-8000 Aarhus C, Denmark; * address correspondence to this author at: Department of Clinical Biochemistry, AKH, University Hospital of Aarhus, Nørrebrogade 44, DK-8000 Aarhus C, Denmark; fax 45-89493060, e-mail vakurbor@hotmail.com)

Competitive binding approaches with use of specific binding proteins are the most commonly used methods to measure vitamin B₁₂ in laboratory medicine. Various binding proteins have been used in these methods, including intrinsic factor (IF), pooled human or chicken serum, transcobalamin (TC), and saliva (1, 2). The most widely used of these are non-human IF preparations, usually obtained from hog gastric mucosa. Numerous problems have been reported with their application, however, including difficulty of purification, instability on storage, and variation in the binding capacity connected with changes in serum protein and vitamin B₁₂ concentrations (1, 2). If IF is not highly purified, it may contain haptocorrins (also called R proteins), which bind not only vitamin B₁₂ but also related metabolically inactive compounds that may be present in the sample, thereby causing artificially increased vitamin B₁₂ results (1, 3).

To circumvent the problems associated with nonhuman IF, we recently expressed human IF in plants and obtained a product free of endogenous vitamin B₁₂ and contaminating vitamin B₁₂-binding proteins (4). In the current study, we examined the feasibility of using this recombinant human IF for measurement of vitamin B₁₂ bound to TC.

Human IF was expressed in the recombinant plant *Arabidopsis thaliana* and purified as described previously (4). As a first step, recombinant human IF was coupled to magnetic beads (Dynabeads, M-280 Tosylactivated; DYNAL) according to the protocol for ligand-binding applications as recommended by the manufacturer. The beads from 1 mL of the standard suspension were washed three times in 0.2 mol/L phosphate-buffered saline, pH 7.4. The washed beads were mixed with 1 mL of recombinant human IF (0.5 g/L) and incubated for 24 h at 37 °C with gentle and continuous agitation. The beads were precipitated, 1 mol/L Tris (pH 7.5) was added to the pellet, and the incubation was then continued for 4 h. The treated beads were then washed five times with 2 mL of 0.05 mol/L Tris (pH 7.5)–0.5 mol/L NaCl and suspended in this buffer.

Recombinant human IF coupled to magnetic beads was used for measurement of vitamin B₁₂ in the last step of the Axis-Shield Holo-TC assay in place of the binding protein supplied with the assay. This step measures the vitamin B₁₂ bound to TC trapped by insolubilized antibodies (5). The Axis-Shield Holo-TC assay was used to demonstrate