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ASSOCIATION OF *UGT1A1* GLY71ARG WITH URINE UROBILINOGEN

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ABSTRACT

Bilirubin is glucoronized by uridine diphosphate-glucuronosyl transferase 1A1 (UGT1A1) mainly in the liver, and excreted into bile. The conjugated form is metabolized into the unconjugated form, and then into urobilinogen by bacteria in the intestine. Unconjugated bilirubin and urobilinogen are absorbed into the blood stream. The kidney filtrates conjugated bilurubin and urobilinogen into urine. Accordingly, the reduced enzyme activity of UGT1A1 may decrease serum conjugated bilirubin levels, resulting in a lower frequency of positive results of urine bilirubin and urobilinogen. This study examined the associations of *UGT1A1* Gly71Arg (*UGT1A1*6*) with urine bilirubin and urobilinogen, as well as serum AST, ALT and GGT. Subjects were 5,172 inhabitants 35 to 69 years old who participated in a cohort study in Nagoya from June 2008 to May 2010. Among them, data from 5,151 participants (1,465 males and 3,686 females) were available for analysis. The age-sex-adjusted odds ratio (OR) of *ArgArg* relative to *GlyGly* was 1.37 (95% confidence interval (95% CI), 0.55–1.23) for bilirubin, and 1.67 (95% CI, 0.86–3.26) for urobilinogen. Those of *ArgArg+ArgGly* were 0.87 (95% CI, 0.59–1.27) and 1.50 (95% CI, 1.17–1.94), respectively. AST, ALT and GGT levels had no associations with the genotype. Although the significant association for urobilinogen was contrary to the biological expectation, this study indicated that *UGT1A1* Gly71Arg may be a genetic factor of urine urobilinogen.

Key Words: Bilirubin, Urobilinogen, UGT1A

INTRODUCTION

Since blood tests are commonly available in ordinary clinical settings for evaluating liver functions with aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the clinical uses for of urine bilirubin and urobilinogen are limited. However, due to their low cost and easiness, urine tests are still options for screening liver functions in some settings, including emergency rooms and health check-up facilites.[1,2] It is not rare for urine bilirubin and urobilinogen to be positive even without liver damage, indicating that some genetic traits, as well as environment factors, may play a role in the positive results among those with normal liver functions.

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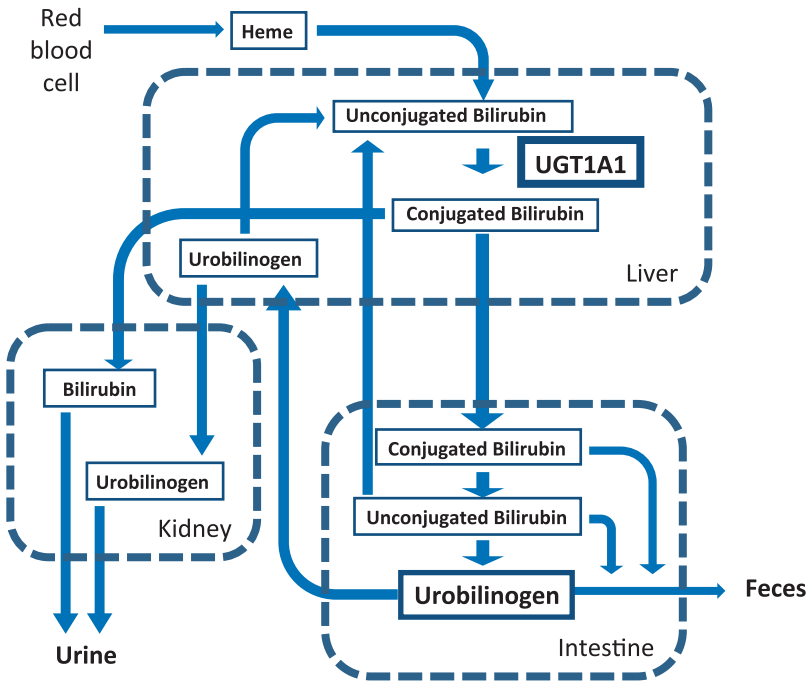

Fig. 1 Pathways on bilirubin and urobilinogen

Uridine diphoshate-glucurosyltransferase (UGT) is a hepatic enzyme that catalyzes the glucuronization of bilirubin into a water-soluble conjugated form to facilitate in its excretion.[3] As shown in Fig. 1, conjugated bilirubin is excreted in bile through the duodenum, and is then unconjugated and oxidized into urobilinogen by enteric bacteria. Unconjugated bilirubin and urobilinogen are excreted in feces. While conjugated bilirubin and urobilinogen are excreted in urine, unconjugated bilirubin is not excreted in urine because it is insoluble in water. The low enzyme activity of UGT may disturb the excretion of conjugated bilirubin and urobilinogen into urine, possibly resulting in a lower frequency of urine bilirubin and urobilinogen under normal conditions.

UGT is encoded by the *UGT1A1* gene at chromosome 2q37.1, whose genotypes with reduced enzyme activity are known to lead to Crigler-Najjar syndrome type 1 (CN-1; severe hyperbilirubinemia), CN-2 (moderate hyperbilirubinemia) and Gilbert's syndrome (GS; mild hyperbilirubinemia).[4] Recent genome-wide association studies showed that serum bilirubin is associated with a genetic variation of the *UGT1A1* locus.[5] Among those with Gilbert's syndrome, the $(TA)_7$ (*UGT1A1*28*) in the promoter region is most common in Caucasians and Africans, while Gly71Arg (*UGT1A1*6*) is more common in Asian populations.[6] In Japan, the $(TA)_7$ allele was reported to be 0.090 among 133 cancer patients treated with irinotecan[7] and 0.066 among 159 newborns,[8] while the *71Arg* allele was 0.226 and 0.195 among cancer patients and newborns, respectively. Among Japanese, the influence of the polymorphisms was reported to be stronger in the *71Arg* allele than in the $(TA)_7$ allele on irinotecan-related toxicity[7] and neonatal hyperbilirubinemia.[8,9]

Urine bilirubin and urobilinogen concentrations were reported to correlate with serum bilirubin.[10] If the *UGT1A1* genotype is associated with urine bilirubin or urobilinogen, urine tests

could be used as predictors for the low enzyme activity genotypes. The present study aimed to examine the associations of the *UGT1A1* Gly71Arg with urine bilirubin and urobilinogen, because the *UGT1A1* Gly71Arg allele appears more frequently and is possibly more influential than *UGT1A1* $(TA)_n$ among Japanese.

MATERIALS AND METHODS

Subjects

Subjects were 5,172 inhabitants of Nagoya who participated in the Daiko Study, a part of the Japan Multi-institutional Collaborative Cohort Study (J-MICC Study).[11] The eligibility criteria for the Daiko Study were: 1) those aged from 35 to 69 years on the day of participation, 2) those having their resident registry inside of Nagoya City on the day of participation, and 3) those who had not been enrolled in other studies affiliated with the J-MICC Study. The Daiko Study started in June 2008 and ended in May 2010. Three participants who had withdrawn from the study before August 31, 2010 were excluded. Data were not available from 18 participants: 16 due to DNA sample unavailability, 1 due to unsuccessful genotyping, and 1 due to urine sample unavailability. Accordingly, 5,151 individuals remained for the analysis. The participants were requested to complete a questionnaire on their lifestyle, to be measured for height, body weight, and abdominal circumference, and to provide urine and blood specimens. The participants were informed of the results of urine (bilirubin, urobilinogen, etc.) and blood (AST, ALT, GGT, etc.) tests. This study was approved by the Ethics Committee of Nagoya University School of Medicine (approval number 618).

Laboratory tests and genotyping

Urinalysis was conducted with Uropaper III "Eiken" (Otsuka Pharmaceutical Co., Ltd., Tokyo). Blood tests were conducted at SRL Co., Ltd., Tokyo.

DNA was extracted from buffy coat conserved at –80°C using a BioRobot® M-48 (QIAGEN Group, Tokyo). *UGT1A1* Gly71Arg (G211A, rs4148323) was genotyped by a polymerase chain reaction with confronting two-pair primers (PCR-CTPP).[12] Each 25 μl reaction tube contained 50–80 ng DNA, 0.12 mM dNTP, 12.5 pmol of each primer, 0.5U AmpliTaq Gold (Perkin-Elmer, Foster City, CA) and 2.5 μl of 10x PCR buffer including 15 mM $MgCl_2$. The PCR was conducted with an initial denaturation at 95°C for 10 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 64°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The primers were F1: 5'-GGA AGA TAC TGT TGA TCC CAG TG-3', R1: 5'-CGT CTT CAA GGT GTA AAA TGC TCC-3', F2: 5'-GCC TCG TTG TAC ATC AGA GAC A-3', and R2: 5'-GTA AGT GGG AAC AGC CAG AC-3'. The amplified DNA fragments were 159-bp for the *Gly* (*G*) allele, 202-bp for the *Arg* (*A*) allele, and 309-bp for the common band. The amplified DNA was visualized on a 2% agarose gel with ethidium bromide staining, as demonstrated in Fig. 2.

Statistical analysis

Hardy-Weinberg equilibrium for genotype frequency was examined with a chi-square test. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using an unconditional logistic regression model. All statistical analysis was performed using STATA Version 7 (STATA Corp. College Station, Texas).

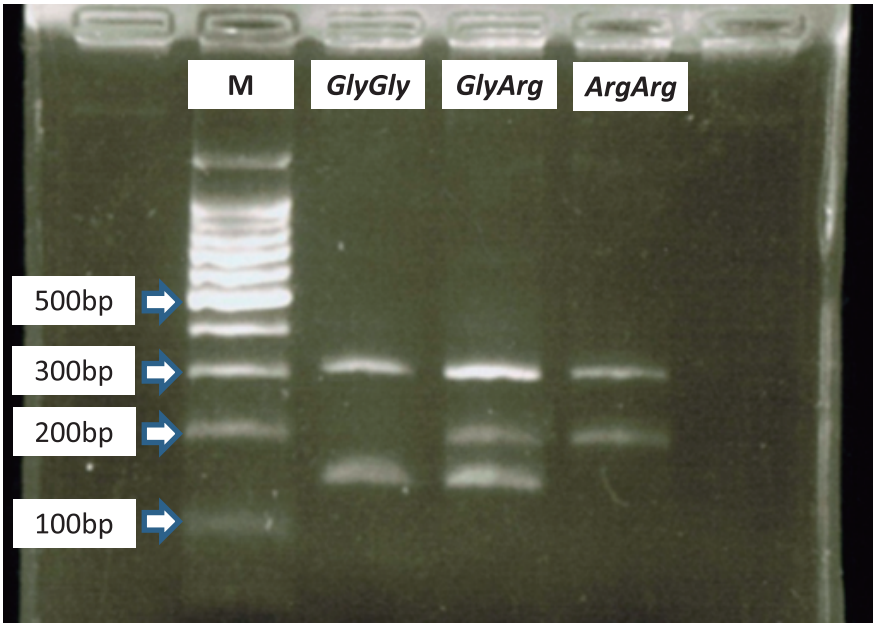

Fig. 2 Agarose gel electrophoresis for *UGT1A1* Gly71Arg polymorphism. Lane M is for a 100-bp DNA ladder, lane *GlyGly* has 159-bp and 309-bp, lane *GlyArg* has 159-bp, 202-bp and 309-bp, lane *ArgArg* has 202-bp and 309-bp.

RESULTS

Characteristics of subjects and genotype frequency

Table 1 shows the characteristics of the 5,151 subjects (1,465 males and 3,686 females). Males aged 60–69 years were relatively numerous, while the age of females distributed almost uniformly across age groups. For men and women, the positives were 3.7% and 2.1% for bilirubin, and 9.3% and 3.6% for urobilinogen, respectively.

The genotype was *GlyGly* for 3,560 participants, *GlyArg* for 1,452 participants, and *ArgArg* for 139 participants, with the result that the *Arg* allele was 0.168 for the present study subjects. The distribution was in Hardy-Weinberg equilibrium (p=0.533).

Associations of UGT1A1 genotype with biomarkers

There was no significant association between the *UGT1A1* genotype and urine bilirubin, as shown in Table 2. The sex-age-adjusted OR for *GlyArg+ArgArg* was 0.87 (95% CI, 0.59–1.27) relative to *GlyGly*. The adjusted OR of urine urobilinogen was 1.49 (95% CI, 1.14–1.94) for GlyArg, 1.67 (95% CI, 0.86–3.26) for *ArgArg*, and 1.50 (95% CI, 1.17–1.94) for *GlyArg+ArgArg*. Table 3 shows the ORs according to sex and age groups. The elevation of the OR was observed only in males. Differences in age did not modify associations. As demonstrated in Table 4, there were no significant associations with serum AST, ALT and GGT.

Table 1 Characteristics of study subjects

Characteristics		Males		Females		Total	
		n	%	n	%	n	%
Age	35–39	191	13.0	571	15.5	762	14.8
	40–49	347	23.7	1,027	27.9	1,374	26.7
	50–59	401	27.4	944	25.6	1,345	26.1
	60–69	526	35.9	1,144	31.0	1,670	32.4
Bilirubin	–	1,410	96.3	3,609	97.9	5,019	97.4
	1+	51	3.5	72	2.0	123	2.4
	2+	3	0.2	5	0.1	8	0.2
	3+	1	0.1	0	0.0	1	0.0
Urobilinogen	–	1,329	90.7	3,555	96.5	4,884	94.8
	1+	108	7.4	112	3.0	220	4.3
	2+	28	1.9	19	0.5	47	0.9
UGT1A1	*GG*	1,010	68.9	2,550	69.2	3,560	69.1
	GA	417	28.5	1,035	28.1	1,452	28.2
	AA	38	2.6	101	2.7	139	2.7
Total		1,465	100	3,686	100	5,151	100

Table 2 Sex-age-adjusted odds ratio (OR) and 95% confidence interval (CI) of *UGT1A1* Gly71Arg for bilirubin and urobilinogen

			UGT1A1 Gly71Arg							
			GlyGly		*GlyArg*		*ArgArg*		*GlyArg+ArgArg*	
Urine test		n	n	%	n	%	n	%	n	%
Bilirubin	–	5,019	3,465	69.0	1,420	28.3	134	2.7	1,554	31.0
	+	132	95	72.0	32	24.2	5	3.8	37	28.0
OR (95% CI)			1 (Reference)		0.82 (0.55–1.23)		1.37 (0.55–3.43)		0.87 (0.59–1.27)	
Urobilinogen	–	4,884	3,399	69.6	1,356	27.8	129	2.6	1,485	30.4
	+	267	161	60.3	96	36.0	10	3.7	106	39.7
OR (95% CI)			1 (Reference)		1.49 (1.14–1.94)		1.67 (0.86–3.26)		1.50 (1.17–1.94)	

"+" includes "1+", "2+" and "3+".

Table 3 Odds ratio (OR) and 95% confidence interval (CI) of *UGT1A1* Gly71Arg for urobilinogen

			UGT1A1 Gly71Arg					
			GlyGly		*GlyArg*		*ArgArg*	
Subjects	Urobilinogen	n	n	%	n	%	n	%
Males	–	1,329	929	69.0	369	27.8	31	2.2
	+	136	81	59.6	48	35.3	7	5.2
	OR (95% CI)		1 (Reference)		1.47 (1.05–2.14)		2.53 (1.08–5.93)	
Females	–	4,484	3,399	75.8	987	22.0	98	2.2
	+	212	161	75.9	48	22.6	3	1.4
	OR (95% CI)		1 (Reference)		1.51 (1.04–2.17)		0.95 (0.29–3.05)	
Age 35–50	–	2,008	229	64.9	114	32.3	10	2.8
	+	128	25	69.4	11	30.6	0	0.0
	OR (95% CI)		1 (Reference)		1.66 (1.14–2.42)		1.86 (0.71–4.89)	
Age 50–69	–	2,876	372	67.5	161	29.2	18	3.3
	+	139	37	68.5	16	29.6	1	1.9
	OR (95% CI)		1 (Reference)		1.34 (0.93–1.94)		1.50 (0.59–3.80)	

Table 4 Sex-age-adjusted odds ratio (OR) and 95% confidence interval (CI) of *UGT1A1* Gly71Arg for AST, ALT and GGT.

			UGT1A1 Gly71Arg					
			GlyGly		*GlyArg*		*ArgArg*	
Blood test		n	n	%	n	%	n	%
AST	<30U/l	4,776	3,295	69.0	1.351	28.3	130	2.7
	≧30U/l	375	265	70.7	101	28.2	9	2.4
	OR (95% CI)		1 (Reference)		0.92 (0.73–1.18)		0.87 (0.44–1.74)	
ALT	<30U/l	4,657	3,213	69.0	1,316	28.3	128	2.8
	≧30U/l	494	347	69.1	136	27.5	11	2.2
	OR (95% CI)		1 (Reference)		0.95 (0.77–1.17)		0.80 (0.42–1.52)	
GGT	<50U/l	4,580	3,153	68.8	129	28.4	128	2.8
	≧50U/l	57	407	71.3	9	26.8	11	1.9
	OR (95% CI)		1 (Reference)		0.92 (0.73–1.11)		0.66 (0.35–126)	

AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: γ-glutamyltranspeptidase

DISCUSSION

Although the *UGT1A1 71Arg* allele with reduced enzymatic activity reduces serum bilirubin levels, this study unexpectedly found that the allele elevated the risk of positive urine urobilinogen, but not urine bilirubin. The association was found only for males, not for females. No associations were observed with ALT, AST, and GGT in blood. The *Arg* allele frequency was 0.168 in a large general population with 5,151 inhabitants of Nagoya City.

The biological explanation for the significant associations with urine urobilinogen was not straightforward. As demonstrated in Fig 1, the reduced enzyme activity of UGT1A1 seemed to reduce the excretion of conjugated bilirubin and the subsequent reduction of urobilinogen synthesis, resulting in a lowered frequency of positive urine urobilinogen. Although there was no biological evidence, possible explanations for the increased risk of urine urobilinogen among those with reduced enzyme activity were: 1) elevation of serum urobilinogen derived from increased unconjugated bilirubin, 2) elevation of urobilinogen synthesis in the intestine, or 3) enhanced urobilinogen excretion from the kidney.

The genotype distribution of *UGT1A1* Gly71Arg was in Hardy-Weinberg equilibrium. The *Arg* allele frequency in this study (0.168 in 5,151 participants) was similar to that of another Japanese study (0.164 in 752 healthy Japanese).[13] The allele frequency was 0.213 in 324 healthy Korean males,[14] slightly higher than among Japanese. HapMap data showed no Europeans and Sub-Saharan Africans with *Arg* allele. Meanwhile, a recent study reported that the *Arg* allele was 0.211 among 109 Turkish newborn infants; the allele was not associated with hyperbilirubinemia.[15]

The genotyping of *UGT1A1* seemed important. At present, **6* (*71Arg*) and **28* ($(TA)_7$) are routinely tested before irinotecan therapy.[16,17] UGT1A1 metabolizes other drugs such as ethinylestradiol, as well as endogenous steroids and xenobiotics including phenols, anthraquinones, and flavones.[18] Thus, knowledge of the genotype seems useful for individuals.

The present study has several limitations. First, the serum bilirubin was not measured to examine the association between serum bilirubin and urine bilirubin. Second, the urine bilirubin was measured by the examiners' judgment using standard color panels for "–," "+," "2+," and "3+," and was not measured quantitatively. Accordingly, adjustments with urine creatinine was not possible. Third, the other polymorphisms of *UGT1A1* with reduced enzyme activity including (TA) n were not genotyped in this study. Fourth, other factors such as constipation were not taken into account in this study. Lastly, subjects may not have been sufficient to detect a weak association

with bilirubin, because bilirubin-positive was relatively rare, even among 5,151 participants.

In conclusion, this study found that *UGT1A1 71Arg* allele elevated the risk of urine urobilinogen, but not urine bilirubin. This association was found only in males. The biological mechanisms involved remain to be elucidated by further studies.

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