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ARTICLE

Substantially Increased Plasma Coproporphyrin-I Concentrations Associated With *OATP1B1*15* Allele in Japanese General Population

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Coproporphyrin-I (CP-I) in plasma is a sensitive and specific endogenous probe for phenotyping organic anion transporting polypeptides 1B (OATP1B, encoded by *SLCO1B*). A few small-scale studies suggested that plasma CP-I concentration is affected by OATP1B1 polymorphism, but detailed studies are lacking. In this large-scale study, we measured plasma CP-I concentrations in 391 subjects from the Japanese general population, and evaluated the relationship between plasma CP-I concentrations and *OATP1B1* polymorphisms to further assess the utility of plasma CP-I concentrations as an endogenous OATP1B probe. Plasma CP-I concentrations were 0.45 ± 0.12, 0.47 ± 0.16, 0.47 ± 0.20, 0.50 ± 0.15, 0.54 ± 0.14, and 0.74 ± 0.31 ng/mL in participants with *OATP1B1*1b/*1b* ($n = 103$), **1a/*1b* ($n = 122$), **1a/*1a* ($n = 40$), **1b/*15* ($n = 74$), **1a/*15* ($n = 41$), and **15/*15* ($n = 11$), respectively, showing an ascending rank order with significant difference ($P < 0.0001$). *Post hoc* analysis revealed significant increases in plasma CP-I concentration in *OATP1B1*1b/*15* ($P = 0.036$), **1a/*15* ($P = 0.0005$), and **15/*15* ($P = 0.0003$) groups compared with the *OATP1B1*1b/*1b* group. There was no significant difference among *OATP1B* genotypes in plasma concentration of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, a uremic toxin reported to decrease OATP1B activity *in vivo*. These findings confirm the utility of plasma CP-I concentrations as an endogenous biomarker for phenotyping of OATP1B activity. Plasma CP-I concentration is potentially useful for the study of drug-drug interactions via OATP1B or individual dose adjustment of OATP1B substrates.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Phenotyping of organic anion transporting polypeptides 1B (OATP1B) seems to be a useful tool for quantitative assessment of *in vivo* OATP1B activity. Several endogenous biomarkers for OATP1B phenotyping have been reported and coproporphyrin-I (CP-I) is especially focused as a sensitive and specific probe.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ Does *OATP1B1*15* allele, which decreases the transporting function of OATP1B1, affect plasma CP-I concentrations in Japanese general population?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ Plasma CP-I concentrations showed an ascending rank order in subjects with *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*, with significant difference. *Post hoc* analysis revealed significant increases in plasma CP-I concentrations in *OATP1B1*1b/*15*, **1a/*15*, and **15/*15* groups compared with the *OATP1B1*1b/*1b* group. Especially, substantial increase was observed in *OATP1B1*15/*15*.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ The results further substantiated the utility of plasma CP-I concentrations as an endogenous biomarker for phenotyping OATP1B activity. Plasma CP-I concentrations can be utilized in studies of drug-drug interactions via OATP1B and individual dose adjustment of OATP1B substrates.

Drug transporters are localized at cell membranes and involved in drug transport in various tissues such as the liver, kidneys, gastrointestinal tract, and brain endothelium. Organic anion transporting polypeptides 1B (OATP1B, encoded by *SLCO1B*) is a hepatic uptake transporter that substantially affect pharmacokinetics of some drugs, such as hydroxymethylglutaryl (HMG)-CoA reductase inhibitors and some anti-hepatitis C virus drugs.[1–6] *In vivo* OATP1B activity has large interindividual variability and is affected by environmental, physiologic, and genetic factors. For example, OATP inhibitors, such as rifampicin and cyclosporin A, inhibit OATP1B activity and increase plasma concentrations

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of OATP1B substrates.[7,8] Chronic kidney failure has been reported to decrease OATP1B activity, and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) is suggested as a causative substance.[9–11] As genetic factors, *SLCO1B1* exhibits two major single nucleotide polymorphisms: A388G and T521C. These polymorphisms form four haplotypes: *OATP1B1*1a* (c.388A-c.521T), *OATP1B1*1b* (c.388G-c.521T), *OATP1B1*5* (c.388A-c.521C), and *OATP1B1*15* (c.388G-c.521C).[1,12] Several studies have revealed that *OATP1B1*5* and *OATP1B1*15* are associated with decreased transporting activities of OATP1B1.[12–16] In the Japanese population, c.521C shows strong linkage disequilibrium with c.388G (r^2 = 0.0708, D' = 0.9999).[1] Thus, *OATP1B1*15* is an important polymorphism that affects individual OATP1B activity *in vivo*.

Phenotyping of OATP1B seems to be a useful tool for quantitative assessment of *in vivo* OATP1B activity. Several probes for phenotyping of OATP1B have been reported, such as probe drugs, including HMG-CoA reductase inhibitors, and endogenous probes, including total and direct bilirubin, chenodeoxycholate 24-glucuronide, glycochenodeoxycholate-3-sulfate, and coproporphyrins.[17,18] Especially coproporphyrin-I (CP-I) and CP-III in plasma are sensitive and specific endogenous probes for phenotyping OATP1B.[19–26] Rifampicin increases plasma CP-I and CP-III concentrations to a greater extent than other endogenous compounds and rosuvastatin.[22] CP-I has been reported to be superior to CP-III as an endogenous OATP1B probe, because CP-I is a selective substrate for OATP1B1 and OATP1B3, whereas CP-III is also a substrate for OATP2B1.[19,20] Thus, quantification of plasma CP-I concentration is a potentially useful tool for dose individualization of OATP1B substrates and assessment of OATP1B-mediated drug-drug interactions.

A few previous small-scale studies suggested that plasma CP-I concentration is affected by OATP1B1 polymorphism, but detailed studies are lacking. Shen *et al*.[25] reported a significant decrease in plasma CP-I concentration in healthy volunteers with SLCO1B1 c.388AG compared with SLCO1B1 c.388AA, but there were no data on haplotypes of SLCO1B1 T521C. Yee *et al*.[27] measured plasma CP-I concentrations in 16 healthy volunteers and found significant difference among 3 groups: SLCO1B1 c.521TT (n = 8), c.521TC (n = 6), and c.521CC (n = 2), but the number of participants were inadequate and data on haplotypes of SLCO1B1 A388G were unknown. The report by Mori *et al*.[28] showed that plasma CP-I concentrations increased in Japanese participants with *OATP1B1*15/*15* compared with those with *OATP1B1*1b/*1b* or *OATP1B1*1b/*15*, but only 2 participants with *OATP1B1*15/*15* were included in the study and statistical analysis was not performed. In the present large-scale study, we measured plasma CP-I concentrations in 391 subjects from the Japanese general population, and evaluated the relationship between plasma CP-I concentrations and *OATP1B1* polymorphisms to further assess the utility of plasma CP-I concentration as an endogenous OATP1B probe.

METHODS

Study participants

We analyzed the data of participants recruited in the Japan Multi-Institutional Collaborative Cohort (J-MICC) Study. The J-MICC study recruited subjects from the general Japanese population, and detected and confirmed gene–environment interactions related to lifestyle-associated diseases using genetic and clinical data.[29] All participants in the J-MICC study gave written informed consent, and the study protocol was approved by the ethics committees at institutions participating in the J-MICC study. We analyzed the data of 500 randomly selected subjects who received a health check in Kyoto Prefectural University of Medicine, and selected 391 participants who met the following inclusion criteria: body mass index lower than 30 kg/m^2, estimated glomerular filtration rate (eGFR) higher than 60 mL/min/1.73 m^2, total bilirubin lower than 1.5 mg/dL, and alanine aminotransaminase lower than 100 IU/L. The eGFR was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation for Japanese.[30] All plasma samples for quantification of CP-I and CMPF were stored in deep freeze (–80°C) and light-resistant condition. This study was approved by the ethics committees of Kyoto Prefectural University of Medicine (approval number: ERB-C-1384) and Meiji Pharmaceutical University (approval number: 3023).

Genotyping

The 14,539 study participants in the J-MICC study from 12 areas of Japan were genotyped at RIKEN Center for Integrative Medicine using a Human OmniExpressExome-8 version 1.2 BeadChip array (Illumina, San Diego, CA, USA).[31] Quality control filtering of samples and single nucleotide polymorphisms (SNPs) have been reported previously.[31] Briefly, for quality control filtering, samples with inconsistent sex information, one of each pair of samples found to be closely related by descent, and samples with ancestry estimated to be outside the Japanese population were excluded. SNPs with a call rate < 0.98 or a Hardy Weinberg equilibrium P value $< 1 \times 10^{-6}$ were also filtered out. Such quality control filtering resulted in the selection of 14,091 subjects and 873,254 SNPs, from whom we selected the above-mentioned 391 participants and their data. Among the SNPs that passed quality control, we identified rs2306283 (c.A388G, p.N130D, and exon 5) and rs4149056 (c.T521C, p.V174A, and exon 5) from genomewide association study data, and formed four haplotypes: *OATP1B1*1a* (c.388A-c.521T), *OATP1B1*1b* (c.388G-c.521T), *OATP1B1*5* (c.388A-c.521C), and *OATP1B1*15* (c.388G-c.521C). Finally, the participants were stratified into six polymorphism groups: *OATP1B1*1b/*1b*, *OATP1B1*1a/*1b*, *OATP1B1*1a/*1a*, *OATP1B1*1b/*15*, *OATP1B1*1a/*15*, and *OATP1B1*15/*15* (**Table 1**). CP-1 may also be transported by other OATPs, such as OATP2B1 (encoded by *SLCO2B1*) and OATP1B3 (encoded by *SLCO1B3*), and also by other types of transporters, such as MRP2 (encoded by *ABCC2*).[19,32] We therefore evaluated the association of plasma CP-1 concentration with the following SNPs: rs717620 (*ABCC2* variant; c.C-24T and 5′UTR),[33] rs12422149 (*SLCO2B1* variant; c.G935A, p.R312Q, and exon 7),[34] rs4149117 (*SLCO1B3* variant; c.T334G, p.S112A, and exon 33),[35] and rs7311358 (*SLCO1B3* variant; c.G699A, p.M233I, and exon 6).[35]

Table 1 OATP1B1 polymorphisms according to the haplotypes

	T521C		
	T/T	**T/C**	**C/C**
A388G			
A/A	**1a/*1a*	-	-
A/G	**1a/*1b*	**1a/*15*	-
G/G	**1b/*1b*	**1b/*15*	**15/*15*

OATP, organic anion transporting polypeptide.

Measurement of plasma concentrations of CP-I and CMPF

Plasma concentrations of CP-I and CMPF were measured simultaneously using an ultra-high performance liquid chromatography coupled to tandem mass spectrometry according to the procedures that we reported previously.[36] Inter-assay and intra-assay accuracy and precision were < 7.6% for CP-I and < 4.1% for CMPF.

Data analysis and statistics

Data are expressed as mean ± SD. The genotype distributions were tested for Hardy–Weinberg equilibrium by χ^2 test. Data were tested for normal distribution using Shapiro–Wilk test. Values in some genotype groups were found to be not normally distributed. Clinical characteristics of participants and plasma CMPF concentrations among groups were compared by Kruskal–Wallis test or χ^2 test. Plasma CP-I concentrations between groups were compared using Kruskal–Wallis test with Dunn's *post hoc* test. Correlation between plasma CP-I and CMPF concentrations was assessed by Spearman's rank correlation coefficient. A P value < 0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism 7 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Participants background

Table 2 shows the laboratory data of the 391 study participants. The genotype distributions of these 2 SNPs conformed to Hardy–Weinberg equilibrium with P values > 0.05 (rs2306283: χ^2 = 0.281 and rs4149056: χ^2 = 0.123). The participants were divided according to OATP1B1 polymorphism into *OATP1B1*1b/*1b* (n = 103), **1a/*1b* (n = 122), **1a/*1a* (n = 40), **1b/*15* (n = 74), **1a/*15* (n = 41), and **15/*15* (n = 11). There were no significant differences in demographic and laboratory variables among genotypes. The eGFR was within the normal range in all groups, with no significant difference among groups.

Plasma CP-I concentrations in six OATP1B1 genotypes

Plasma CP-I concentration (mean ± SD) in all participants was 0.48 ± 0.17 (range 0.13–1.41) ng/mL, showing large individual variability. When stratified by *OATP1B1* genotype, plasma CP-I concentrations were 0.45 ± 0.12, 0.47 ± 0.16, 0.47 ± 0.20, 0.50 ± 0.15, 0.54 ± 0.14, and 0.74 ± 0.31 ng/mL in participants with *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*, respectively, showing an ascending rank order with a significant difference detected

Table 2 Characteristics of participants divided into *OATP1B1* genotypes

Characteristic	***OATP1B1*1b/*1b***	***OATP1B1*1a/*1b***	***OATP1B1*1a/*1a***	***OATP1B1*1b/*15***	***OATP1B1*1a/*15***	***OATP1B1*15/*15***	***P* value**
No. of participants	103	122	40	74	41	11	
Males/ females	32/71	37/85	12/28	17/57	10/31	4/7	NS
Age, year	55.9 ± 11.0 [40–74]	55.3 ± 10.0 [39–74]	56.9 ± 9.1 [39–72]	56.0 ± 9.3 [40–73]	58.2 ± 8.7 [39–74]	56.6 ± 10.2 [41–68]	NS
BMI, kg/m^2	21.9 ± 2.8 [15.4–29.9]	22.3 ± 3.0 [14.5–29.8]	22.1 ± 2.9 [17.4–28.0]	21.7 ± 3.4 [15.8–29.7]	21.4 ± 2.4 [17.6–29.5]	21.9 ± 3.6 [16.2–27.1]	NS
eGFR, mL/ min/1.73 m^2	77.3 ± 8.4 [60.0–96.3]	78.6 ± 9.1 [60.7–94.9]	78.9 ± 8.4 [60.4–92.7]	79.9 ± 8.0 [60.5–94.7]	78.8 ± 7.6 [60.7–90.6]	80.1 ± 6.5 [74.0–94.2]	NS
Total bilirubin, mg/dL	0.79 ± 0.24 [0.4–1.4]	0.81 ± 0.24 [0.4–1.4]	0.70 ± 0.19 [0.4–1.0]	0.74 ± 0.22 [0.3–1.3]	0.78 ± 0.22 [0.3–1.3]	0.77 ± 0.28 [0.4–1.2]	NS
ALT, IU/L	17.5 ± 9.4 [5–59]	17.5 ± 9.7 [6–53]	20.5 ± 10.1 [10–53]	19.7 ± 12.4 [7–99]	16.0 ± 6.7 [8–49]	26.1 ± 18.3 [9–61]	NS
Plasma CP-I concentrations, ng/mL	0.45 ± 0.12 [0.21–0.88]	0.47 ± 0.16 [0.13–1.22]	0.47 ± 0.20 [0.19–1.41]	0.50 ± 0.15 [0.21–0.95]	0.54 ± 0.14 [0.25–0.84]	0.74 ± 0.31 [0.44–1.37]	$p < 0.0001$

Data are expressed as numbers, or mean ± SD [range].
ALT, alanine aminotransaminase; BMI, body mass index; CP-I, coproporphyrin-I; eGFR, estimated glomerular filtration rate; NA, not significant; OATP1B1, organic anion transporting polypeptides 1B1.

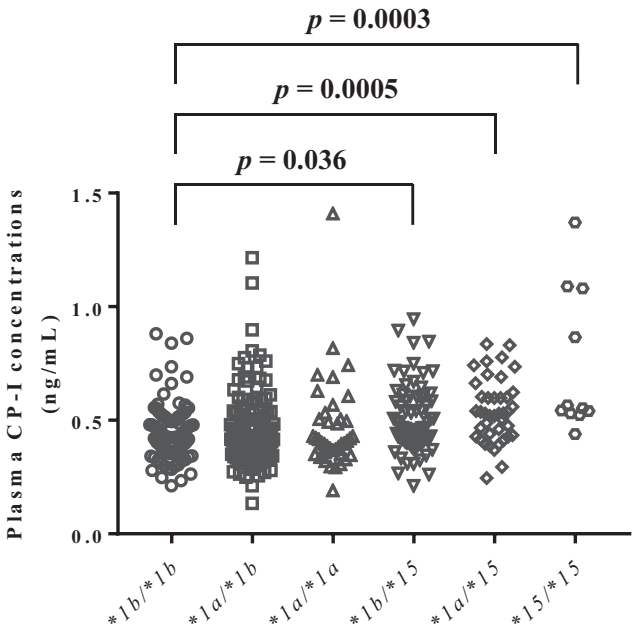

Figure 1 Plasma coproporphyrin-I (CP-I) concentrations in participants with organic anion transporting polypeptide (*OATP*)*1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*. Data were analyzed by Kruskal–Wallis test ($P < 0.0001$) followed by Dunn's *post hoc* test.

by Kruskal–Wallis test ($P < 0.0001$; **Figure 1**). *Post hoc* analysis revealed significant increases in plasma CP-I concentrations in *OATP1B1*1b/*15*, **1a/*15*, and **15/*15* groups compared with *OATP1B1*1b/*1b* group (**Figure 1**).

Plasma CP-I concentrations in additional transporter genotypes

Figure 2 shows the association of plasma CP-I concentrations with genotypes of four additional transporter genes. There was no significant difference among three genotypes in rs717620 (MRP2), rs12422149 (OATP2B1), rs4149117 (OATP1B3), and rs7311358 (OATP1B3).

Relationship between plasma CP-I and CMPF concentrations

Plasma CMPF concentrations were 2,739 ± 1,591, 2,583 ± 1,384, 2,684 ± 1,582, 2,508 ± 1,210, 2,445 ± 1,745, and 2,660 ± 1,461 ng/mL in participants with *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*, respectively, with no significant difference (**Figure 3**). **Figure 4** shows the relationship between plasma CP-I and CMPF concentrations for each *OATP1B1* genotype. No significant correlation was observed for all genotypes.

DISCUSSION

In 2016, CP-I was reported for the first time as a useful endogenous biomarker for phenotyping of OATP1B activity.[19–21] Currently, CP-I is assumed to be the most sensitive and specific biomarker for phenotyping of OATP1B activity compared with the other endogenous biomarkers and probe drugs, such as HMG-CoA reductase inhibitors.[22] In fact, CP-I was used for OATP1B activity phenotyping in clinical drug-drug interaction studies[24,37–39] and in model-based analysis of drug-drug interactions.[23,40–42] Three previous studies reported the relationship between plasma CP-I concentrations and *OATP1B1* polymorphism, but the

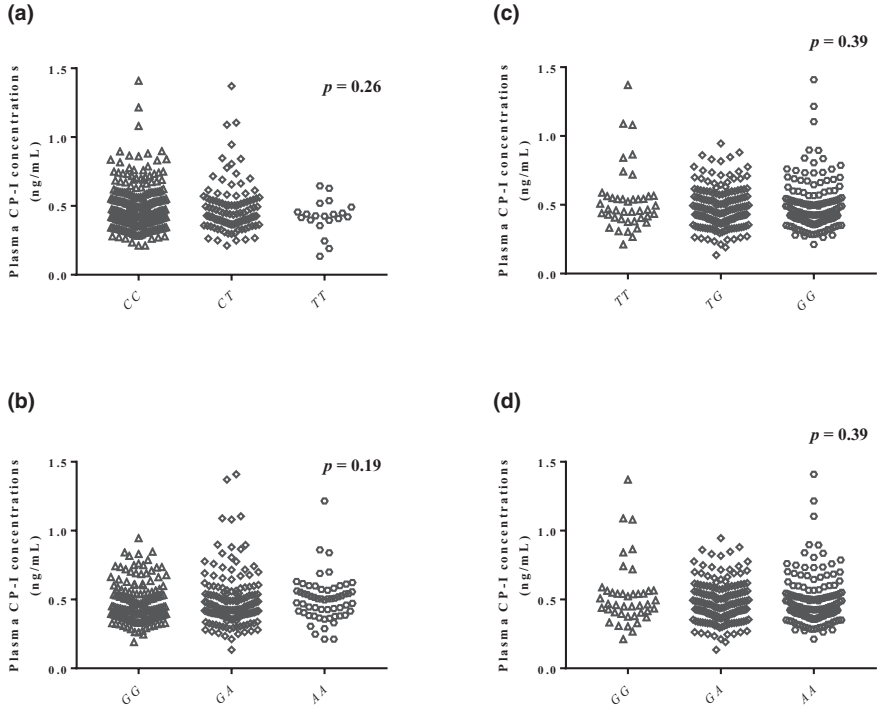

Figure 2 Relationship between plasma coproporphyrin-I (CP-I) concentration and genotypes of rs717620 (**a**), rs12422149 (**b**), rs4149117 (**c**), and rs7311358 (**d**). The rs4149117 **c** and rs7311358 **d** demonstrate complete linkage disequilibrium.

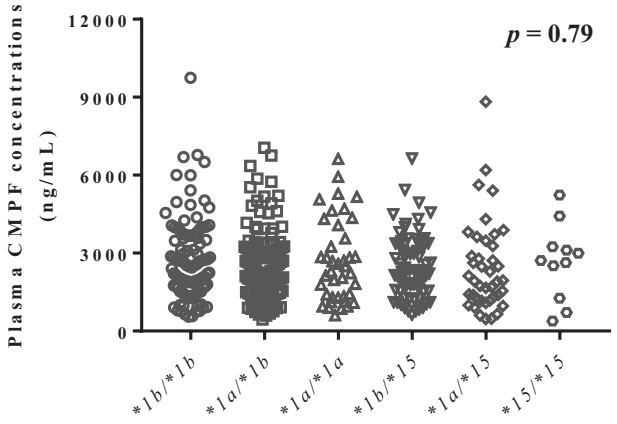

Figure 3 Plasma 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) concentrations in participants with organic anion transporting polypeptide (*OATP*)*1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*. Data were analyzed by Kruskal–Wallis test.

numbers of participants in all studies were insufficient to detect significant differences among the genotypes.[25,27,28] In the present study of a large sample from the Japanese general population, we evaluated the differences in plasma CP-I concentrations in participants with *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, or **15/*15* to evaluate the utility of plasma CP-I concentrations.

Hepatic failure decreased mRNA levels of OATPs in liver biopsy specimens in a previous study.[43] Chronic kidney disease was also reported to decrease OATP1B activity in patients.[9–11] Furthermore, polycystic kidney disease decreased the protein expression of OATPs in rats.[44] In our study, all of the 391 participants had normal values of eGFR, total bilirubin, and alanine aminotransaminase, suggesting that plasma CP-I concentrations in this study were not affected by disease states, such as hepatic and renal failure, but were influenced mainly by OATP1B1 polymorphisms. We evaluated four haplotypes: *OATP1B1*1a*, *OATP1B1*1b*, *OATP1B1*5*, and *OATP1B1*15*. SLCO1B1 c.521C shows strong linkage disequilibrium with SLCO1B1 c.388G in the Japanese population (r^2 = 0.0708, D' = 0.9999).[1] Therefore, we evaluated the influence of 6 types of polymorphism; *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*, on plasma CP-I concentrations. The allele frequency of *OATP1B1*15* in the Japanese population was reported to be 0.11–0.16,[45] which is consistent with the finding in this study. Our study population included 11 participants with *OATP1B1*15/*15*, and this is the largest number

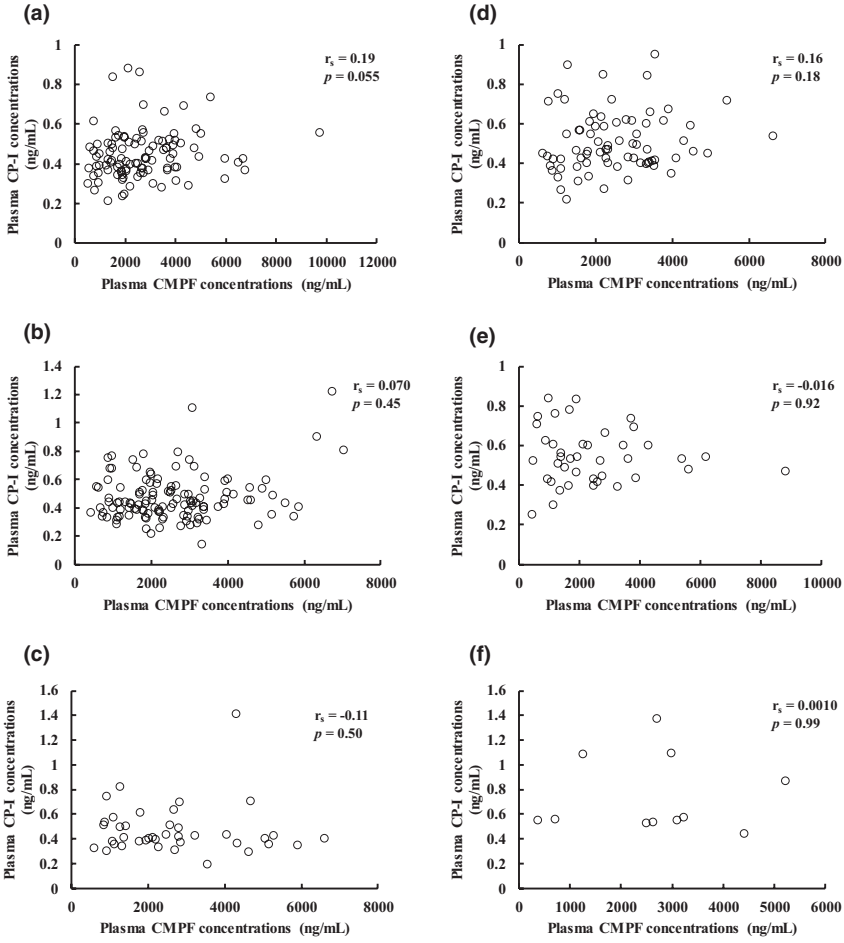

Figure 4 Correlation between plasma coproporphyrin-I (CP-I) and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) concentrations for each organic anion transporting polypeptide (OATP)1B1 genotype.

compared with previous studies that evaluated OATP1B1 polymorphism and plasma CP-I concentrations.[25,27,28] The Japanese population has higher allele frequency of *OATP1B1*15* compared with white, African American, and other Asian populations.[45,46] Thus, the Japanese population seems to be suitable for the purpose of this study.

We obtained several important findings in this study. First, significant increases in plasma CP-I concentration were observed in *OATP1B1*1b/*15*, **1a/*15*, and **15/*15* groups compared with the *OATP1B1*1b/*1b* group. Especially, substantial increase was observed in *OATP1B1*15/*15*. These findings suggest that plasma CP-I concentrations reflect the individual variation of OATP1B activity caused by *OATP1B1* polymorphism. Second, we observed an ascending rank order of plasma CP-I concentrations in subjects with *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15*. This trend is rational considering previous reports showing that the *OATP1B1*1b* allele increases the transportation function of OATP1B1,[47] whereas the *OATP1B1*15* allele decreases transportation function compared with wild type (*OATP1B1*1a*).[12–16] On the other hand, Choi *et al*.[48] studied the effect of OATP1B1 polymorphisms on rosuvastatin pharmacokinetics, and reported that mean areas under the curve of rosuvastatin in *OATP1B1*1b/*1b*, **1a/*1b*, **1a/*1a*, **1b/*15*, **1a/*15*, and **15/*15* were 116, 126, 87.4, 105, 167, and 191 ng h/mL, respectively, which showed no consistent pattern, unlike the ascending trend found in the present study. These results suggest that plasma CP-I concentrations may have better specificity than rosuvastatin as an OATP1B probe. Third, polymorphisms of MRP2, OATP2B1, and OATP1B3 were not associated with plasma CP-I concentrations in this study. This suggests that CP-I is a more sensitive endogenous probe for OATP1B1 than other transporters. Finally, there was no significant difference in plasma CMPF concentrations among the *OATP1B1* genotypes, and no significant correlation between plasma CMPF and CP-I concentrations in all genotypes, suggesting that the variation in CP-I concentration was not due to recruitment of subjects with chronic kidney disease. CMPF has been reported to decrease OATP1B activity in a concentration–dependent manner, both *in vitro*[9] and in clinical studies,[10,36] which further support the absence of effect of CMPF in our study and that the effect of OATP1B1 polymorphism on plasma CP-I concentrations was evaluated appropriately. These findings further demonstrate the utility of plasma CP-I concentration as an endogenous biomarker for phenotyping of OATP1B activity. Plasma CP-I concentrations is useful for studies of drug-drug interaction via OATP1B and individual dose adjustment of OATP1B substrates.

In this large-scale study in the Japanese general population, the mean plasma CP-I concentration was 0.48 ng/mL. This value was slightly lower compared with previous studies in the white population (mean concentration: 0.60–0.71 ng/mL).[49] The difference might be due to genetic differences, such as OATP1B1 polymorphism, but the detail mechanism is unknown. Further studies are needed to reveal the racial differences of plasma CP-I concentrations.

There was a limitation in this study. We measured plasma CP-I concentrations in this study, but did not measure other endogenous probes, such as direct bilirubin, chenodeoxycholate 24-glucuronide, and glycochenodeoxycholate-3-sulfate. Further studies are needed to evaluate the relationship of OATP1B1 genotypes with these probes.

In conclusion, significant differences in plasma CP-I concentrations were observed among *OATP1B1* genotypes, and the concentrations were significantly increased in *OATP1B1*1b/*15, *1a/*15* and **15/*15* compared with *OATP1B1*1b/*1b*. These findings further substantiate the utility of plasma CP-I concentrations as an endogenous biomarker for phenotyping of OATP1B activity.

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Conflicts of Interest. The authors declared no competing interests for this work.

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