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Inhibitory Effect of 5β-Pregnane-3α,20β-Diol on Transcriptional Activity and Enzyme Activity of Human Bilirubin UDP-Glucuronosyltransferase

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ABSTRACT: Prolonged unconjugated hyperbilirubinemia in infants associated with breast milk feeding is a common pediatric problem known as breast milk jaundice (BMJ). A polymorphic mutation (G71R) of bilirubin UDP-glucuronosyltransferase (UGT1A1) is a known cause of BMJ on the infantile side, but the responsible components of breast milk are not currently known. We analyzed the inhibitory effect of 5β-pregnane-3α,20β-diol (pregnanediol) on transcriptional activity and enzyme activity of UGT1A1. To this end, we constructed two types of expression vectors. One type comprised vectors including the upstream enhancer-promoter sequence of *UGT1A1* for WT and variant type (-3279T>G with A(TA)7TAA), used in studying transcriptional regulation. The other type comprised vectors including cDNA of UGT1A1 for WT and the G71R variant, used in studying enzyme activity. In an *in vitro* expression study, pregnanediol did not affect the transcriptional activity of *UGT1A1* enhancer-promoter complex of WT and variant type, even with coexistence of transcriptional factors such as constitutive androstane receptor or pregnane X receptor. In contrast, in the presence of 100 μM pregnanediol, bilirubin glucuronidation of G71R-UGT1A1 was reduced to 51% of WT. We suggest that pregnanediol is a cause of breast milk jaundice in carriers of G71R. **(*Pediatr Res* 70: 453–457, 2011)**

Breast milk jaundice (BMJ) is characterized by prolonged unconjugated hyperbilirubinemia in healthy infants connected with ingestion of breast milk (1). Infants with BMJ always have an elevated serum bilirubin level, above 171 μM (10 mg/dL); it occasionally exceeds 342 μM (20 mg/dL), and at such levels infants are at risk of bilirubin encephalopathy (kernicterus) (2).

BMJ was first described by Newman and Gross, who found a causal relation between human milk feeding and jaundice, based on rapid clearance of jaundice when the infants were taken off breast milk (3). Arias *et al.* (4) first reported the inhibiting effect of breast milk on human liver slices and homogenates using bilirubin and *o*-aminophenol as substrates for conjugation. In their pioneering study, the inhibitory substance in milk was identified as 5β-pregnane-3α,20β-diol (pregnanediol). However, a further study by Adlard *et al.* (5) found that bilirubin conjugation by slices taken from two human livers was not inhibited by pregnanediol. Moreover, other research groups have reported that BMJ may be caused by other breast milk components such as free fatty acids (FFA) or β-glucuronidase (6,7). The responsible substance in breast milk has not yet been identified with certainty (8–10).

The elimination pathway of bilirubin in humans is catalyzed exclusively by bilirubin UDP-glucuronosyltransferase (UGT1A1) (11). A defect in UGT1A1 causes hereditary unconjugated hyperbilirubinemias, specifically Crigler-Najjar syndrome type I (CN-I; MIM #218800), Crigler-Najjar syndrome type II (CN-II; MIM #606785), and Gilbert syndrome (GS; MIM #43500) (12–16). Polymorphic mutations of UGT1A1, namely G71R in the coding region and A(TA)7TAA with -3279T>G in the regulatory region, are the most common causes of Gilbert syndrome, and the latter linked mutations are the sole cause of Gilbert syndrome in Caucasians and Africans (17). Maruo *et al.* (18,19) have shown that G71R (but not A(TA)7TAA with -3279T>G) is an infantile cause of prolonged unconjugated hyperbilirubinemia in East Asia and is also a risk factor for BMJ.

Previous studies seeking substances in breast milk that lead to BMJ were based on histochemical and enzymatic assays using samples prepared from tissues. In the present study, we analyzed the effect of pregnanediol on both wild and variant UGT1A1s based on our gene analyses of infants suffering from neonatal and BMJ. We examined the effect of pregnanediol on the transcriptional activities of *UGT1A1* of both WT (-3279T with A(TA)6TAA) and variant-type (-3279G with A(TA)7TAA) enhancer-promoter complexes. The variant-type enhancer-promoter is the cause of Gilbert syndrome in Caucasian and African populations (17). We also examined the effect of pregnanediol on transcriptional activities of UGT1A1 in the presence of constitutive androstane receptor (CAR) and pregnane X receptor (PXR), which have both been proposed as key transcriptional factors of bilirubin clearance in the human liver (20–24). We first examined the effect of pregnanediol on enzyme activities of WT UGT1A1 and G71R-UGT1A1 in an *in vitro* study.

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Abbreviations: BMJ, breast milk jaundice; **CAR,** constitutive androstane receptor; **MEM,** minimal essential medium; **PXR,** pregnane X receptor; **UGT1A1,** bilirubin UDP-glucuronosyltransferase

METHODS

Effect of pregnanediol on transcriptional activity of UGT1A1. To construct an expression vector, two types of enhancer-promoter complex, including upstream of UGT1A1 (c.-1 to c.-4076); WT enhancer-promoter complex and variant-type enhancer-promoter complex (-3279 T>G with A(TA)7TAA) (variant-type enhance-promoter) of DNA fragments, were amplified from genomic DNA. The distal region (c.-4076 to c.-1945) and the proximal region (c.-2483 to c.247) were amplified separately by PCR using the respective primers with the restriction sites: 5′-GAGAGCTCCCTCAGCCCCTAGAGCACCATC-3′/ 5′-CTCTCGAGGCGCCTTTGCTCCTGCCAGAGG-3′ (the *Sac*I site is underlined) and 5′-GAGAGCTCGAAGGGATTAGTTTAGGACAACCCTCCTTC-3′/ 5′-GCAGAGCTTCCAAGCTTTTTGAGGCTG-3′ (the *Xho*I site is underlined).

Each PCR product (proximal; c.2323 to c.-1; distal:c.-4076 to c.-1945) was inserted into the pCR-XL-TOPO vector using a TOPO XL PCR cloning kit. We constructed PGV-B2 expression vectors (TOYO B-Net Co., Ltd, Tokyo, Japan) having WT enhancer-promoter and variant type enhancer-promoter with continuous 4-kbp regulatory region (c.-4076 to c.-1) by restriction (*Sac*I and *Xho*I) and ligation using a Ligation-Convenience Kit (NIPPON GENE Co., Ltd, Tokyo, Japan) as described previously (25).

CAR-cDNA was amplified from a human liver cDNA library (TaKaRa BIO, Inc.) by PCR using the following primers with the restriction sites (*Eco*R I and *Eco*R V): 5′-GATCGAATTCGTCATGGCCAGTGGGAAGAT-GAG-3′ (the *Eco*R I site is underlined) and 5′-GATCGATATCTCAGCTG-CAGATCTCCTGGAGCCAG-3′ (the *Eco*R V site is underlined) (26). The PCR product ligated into the pCR3.1 vector (Invitrogen Corporation). PXR-cDNA was amplified from a human liver cDNA library (TaKaRa BIO, Inc.) by PCR using the following primers: 5′-AGCCATGAATTCCTGGAGGT-GAGACCCAAA-3′ and 5′-AGCCATGAATTCTCAGCTACCTGTGAT-GCC-3′ (the *Eco*R I site is underlined) (27). PXR-cDNA was cut out by *Eco*R I. The PCR product was ligated into the pcDNA 3.3-TOPO vector (Invitrogen Corporation).

The *in vitro* expression study by assay for transcriptional activity of WT enhancer-promoter and variant-type enhancer-promoter (c.-4076 to c.-1) proceeded as we have reported elsewhere (26), with minor modification. At 25 h before transfection, 7×10^4 HepG2 cells (Riken Cell Bank, Japan) cultured in minimal essential medium (MEM; Invitrogen Corporation) with 10% fetal bovine serum (Invitrogen Corporation) were seeded onto 24-well culture plates.

For transfection, 250 μL of MEM, 3.0 μL of GenePORTER transfection reagent (GeneLantis, San Diego, CA), and plasmid vectors were poured onto the cells in each well. The composition of the plasmid vectors for transfection was as follows: luciferase reporter plasmid PGV-B2 with the transcriptional regulatory region (400 ng), the CAR-pCR3.1 expression plasmid or PXR-pCR3.1 expression plasmid (200 ng), and the Renilla luciferase expression plasmid pRL-SV40 (6 ng; TOYO B-Net Co., Ltd) for normalization. After 3 h, 250 μL of MEM containing 20% fetal bovine serum and 0 to 100 mM pregnanediol was added to each well. After 25 h, the transfected cells in each well were lysed, and the dual luciferase activities were immediately measured with a PicaGene Dual SeaPansy Luminescence Kit (TOYO B-Net Co.).

Effect of pregnanediol on bilirubin glucuronidation activity of UGT1A1. The cDNA of human liver *UGT1A1* was amplified from a human cDNA library (TaKaRa BIO, Inc.) by PCR using the primer pair 5′-CATGGCTGTG-GAGTCCCAGGGC-3′ and 5′-GAATCCCGCACTCCCAAACAGG-3′ and was inserted into a pCR3.1 expression vector using a eukaryotic bidirectional TA cloning kit (Invitrogen, San Diego, CA). The constructed WT cDNA was excised from the pCR3.1 vector by two restriction enzymes (*Hind*III and *Xba*I) and ligated into a pkF18 vector (TaKaRa BIO, Inc.) for mutagenesis. To introduce the mutations into the *UGT1A1* cDNA, we used the following primers (the mutation point is underlined): 5′-CATCAGAGACAGAG-CATTTTA-3′ for the G to A transversion at nucleotide 211 (c.211G>A) for G71R using the site-directed mutagenesis method and a Mutan Km Kit (TAKARA, Kyoto, Japan). The converted cDNA was cut out and religated into the pCR3.1 vector. We verified the cDNA by sequencing (18).

At 24 h before transfection, 6×10^5 COS-7 cells suspended in DMEM with 10% fetal bovine serum were seeded onto 100-mm-diameter culture plates. For transfection, we poured onto the cells 5 mL of DMEM, containing 50 μL GenePORTER Transfection Reagent (GeneLantis) and 10.0 μg DNA. Four hours later, 5 mL of DMEM containing 20% fetal bovine serum was added to each plate. Two models were generated, WT UGT1A1 and G71R-UGT1A1, and nontransfected cells were used as controls. After 48 h, the transfected cells were harvested and stored at −80°C before use. Protein content was measured with a Bicinchoninic Acid Protein Assay Kit (Pierce Chemical, Rockford, IL).

The methods used in the assay for glucuronidation activity toward bilirubin by UGT1A1 have been reported previously (28). The glucuronidation process of the substrate was assayed with [^{14}C] UDP-glucuronic acid (PerkinElmer Life and Analytical Sciences, Boston, MA). The incubation mixture contained 100 to 150 μg of cell homogenate, 0 to 400 μM unconjugated bilirubin (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 500 μM UDP-glucuronic acid (Sigma Chemical Co.-Aldrich, St. Louis, MO), 9.25 kBq [^{14}C] UDP-glucuronic acid (5.97 μM), DMSO (1%), 5 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.5), and 5 mM saccharolactone in a final volume of 100 μL. Incubation took place for 30 min at 37°C. The resultant bilirubin-glucuronide was isolated by thin layer chromatography (TLC) on a TLC plastic sheet 5748 (Merck, Darmstadt, Germany) and was scanned on an Instant Imager (Packard, Meriden, CT). At a bilirubin concentration of 400 μM, pregnanediol (0–100 μM) was added to the reaction mixture to examine its inhibitory effect on bilirubin glucuronidation.

The amounts of enzyme expressed were determined by Western blotting analysis, using anti-UGT1A antibody (diluted 1:10,000; BD Gentest, Woburn, MA) to adjust the enzyme activity. The cell homogenates underwent SDS-PAGE. The protein was transferred to a polyvinylidene fluoride membrane. The membrane was incubated for 4 h in blocking solution, 1 h in a solution of rabbit anti-human UGT1A (diluted 1:10,000; BD Gentest), and 1 h in a solution of anti-rabbit antibody (1:20,000). The detection solution was then added, and the membrane was exposed to film for 5 min, after which the protein was visualized (ECL: enhanced chemiluminescence Plus Western blotting detection system; Amersham Bioscience, United Kingdom). The relative amounts of UGT1A1 expressed at the protein bands were measured with a LAS-4000 (Fujifilm Lifescience, Tokyo, Japan).

Data analysis. We calculated kinetic parameters by performing nonlinear regression on the Michaelis-Menten equation using the Prism 3.0 software (Graph Pad Software, San Diego, CA). Changes in transcriptional activity and enzyme activity were analyzed by regression analysis using JMP software (SAS Institute, Inc., NC).

RESULTS

Effect of pregnanediol on transcriptional activity of UGT1A1. Transcriptional activity of the regulatory region of *UGT1A1* with variant-type enhancer-promoter decreased to 42% of WT enhancer-promoter ($p < 0.05$). The transcriptional activity of WT and variant type was not reduced in the presence of pregnanediol (up to100 μM); Figure 1.

As shown in Figure 2, the transcriptional activities of the regulatory region with WT and variant type were increased about 4-fold in the presence of CAR ($p < 0.001$). Transcriptional activities of both of the enhancer-promoter complexes were not inhibited by pregnanediol, even in the presence of CAR.

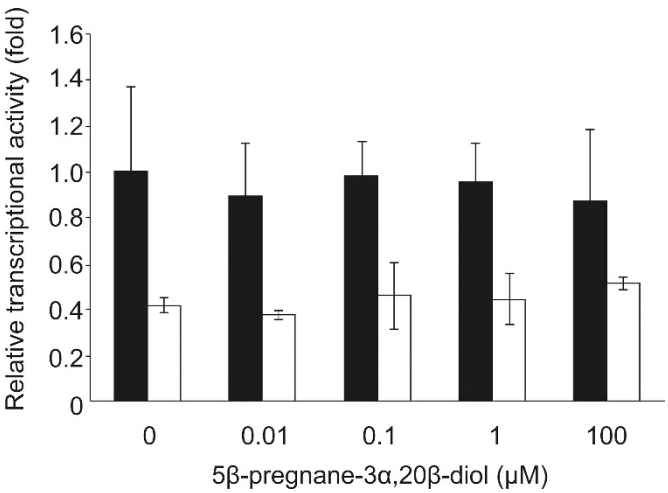

Figure 1. Effect of pregnanediol on transcriptional activities of the regulatory region (c.4076 to c.-1) of *UGT1A1*; WT enhancer-promoter (■) and variant-type enhancer-promoter [-3279T>G with A(TA)7TAA] (□). All activities in the figure are expressed relative to the mean transcriptional activity of WT in the absence of pregnanediol, taken as 1.00.

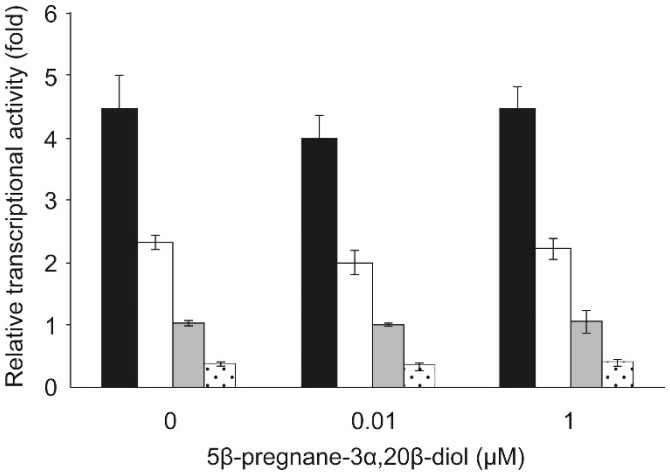

Figure 2. Effect of pregnanediol on transcriptional activities of the regulatory region (c.4076 to c.-1) of WT and variant-type *UGT1A1* in the presence of CAR. Bars are as follows: WT (■), variant type (□), WT with CAR (▩), and variant type with CAR (⚄). All activities in the figure are expressed relative to the mean transcriptional activity of WT in the absence of pregnanediol, taken as 1.00.

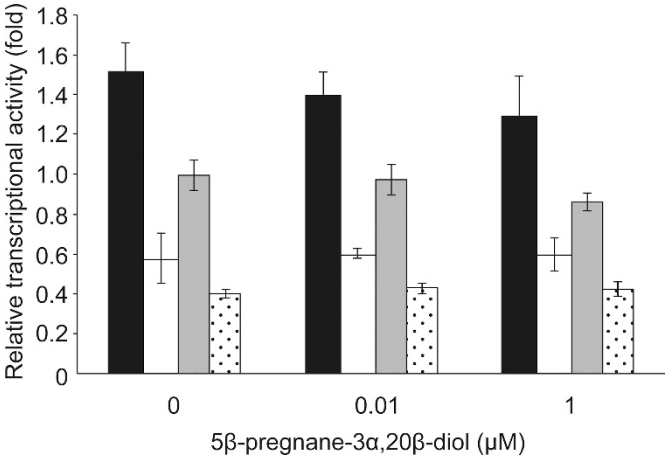

Figure 3. Effect of pregnanediol on transcriptional activities of the regulatory region (c.4076 to c.-1) of WT and variant-type *UGT1A1* in the presence of PXR. Bars are as follows: WT (■), variant type (□), WT with PXR (▩), variant type with PXR (⚄). All activities in the figure are expressed relative to the mean transcriptional activity of WT in the absence of pregnanediol, taken as 1.00.

In the presence of PXR, the transcriptional activities of the regulatory region with WT and variant type increased by a factor of about 1.5 ($p < 0.001$); Figure 3. As with CAR, pregnanediol did not inhibit the transcriptional activities of either of the enhancer-promoter complexes in the presence of PXR.

Effect of pregnanediol on bilirubin glucuronidation of UGT1A1. The relative expression levels of each UGT1A1 protein were determined by Western blotting with a polyclonal anti-human UGT1A antibody. Simultaneous expression of wild UGT1A1 in three independent experiments resulted in UGT1A1 protein levels in the culture cells between 0.9 and 1.1 times the average amount. The amount of G71R UGT1A1 was always 0.85–0.9 times that of wild.

Table 1 shows the kinetic parameters of WT UGT1A1 and G71R-UGT1A1. The V_{max} value of WT UGT1A1 was 7.80 pmol/min/mg protein. For G71R-UGT1A1, V_{max} was 83.3%

Table 1. *Kinetic parameters of WT and G71R UGT1A1 for bilirubin glucuronidation*

	WT UGT1A1	G71R UGT1A1
*V*max (pmol/min/mg protein)	7.802	6.504*
*K*m (μM)	10.61	6.202

* The Vmax value of G71R UGT1A1 is 83.3% of WT UGT1A1.

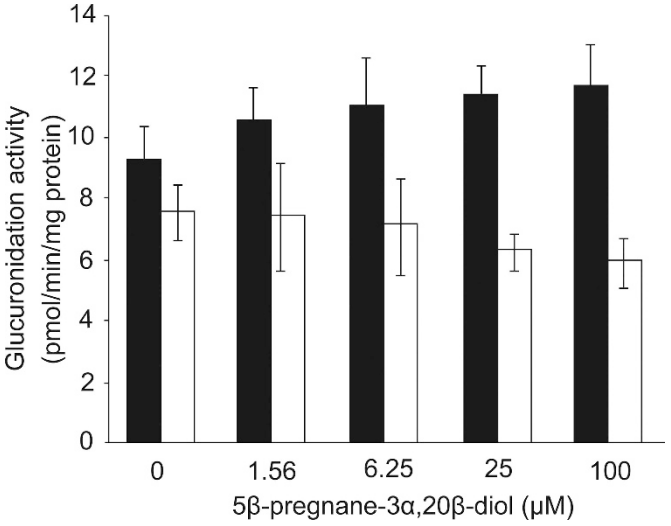

Figure 4. Effect of pregnanediol on bilirubin glucuronidation of WT (■) and G71R-UGT1A1(□). Bilirubin glucuronidation of UGT1A1s was assayed at 400 μM bilirubin in the presence of pregnanediol (0 to 100 μM).

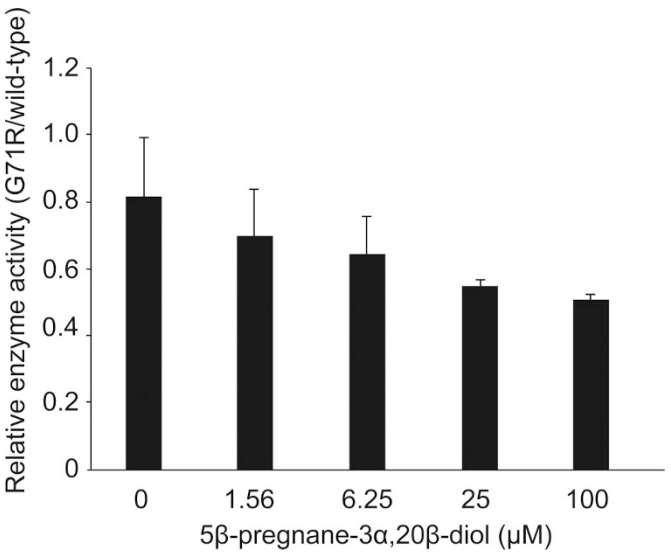

Figure 5. Relative enzyme activity (G71R/WT) of bilirubin glucuronidation in the presence of pregnanediol (0 to 100 μM).

of WT. The enzyme activity for bilirubin glucuronidation of WT UGT1A1 in the absence of pregnanediol was 9.34 ± 1.07 pmol/min/mg protein and that of G71R-UGT1A1 was 7.57 ± 0.90 pmol/min/mg protein (81.0% of WT); see Figure 4. Unexpectedly, the enzyme activity of WT increased slightly on adding pregnanediol, in all concentrations (Fig. 4). In contrast, the enzyme activity of G71R-UGT1A1 decreased slightly in the presence of pregnanediol in all concentrations. The ratio of the activity of G71R-UGT1A1 to WT decreased from 81% to 51% on adding 100 μM pregnanediol (Fig. 5).

This ratio decreased significantly in the presence of pregnanediol according to our regression analysis ($p = 0.02$).

DISCUSSION

BMJ is characterized by prolonged unconjugated hyperbilirubinemia associated with breast milk feeding in healthy infants more than 3–4 wk after birth. BMJ has been variously ascribed to breast milk components such as 5β-pregnane-3α,20β-diol (pregnanediol), other steroids (cortisol and estriol), nonesterified fatty acids, and β-glucuronidase, but the relevant components of breast milk have not yet been identified.

We have investigated here the inhibition of transcriptional activity of *UGT1A1* by pregnanediol using an *in vitro* expression system. The transcriptional activities of WT and variant-type (-3279T>G with A(TA)7TAA) enhancer-promoter were both unaffected by pregnanediol (Fig. 1). The gtPBREM (phenobarbital-responsive enhancer module) in the regulatory region of *UGT1A1* is an important enhancer module of *UGT1A1*, and functions in the presence of nuclear receptors (29,30) such as CAR and PXR, which have been proposed as key transcriptional factors of bilirubin clearance in the human liver and intestine (31). The transcriptional activities of WT and variant-type enhancer-promoters both increased in the presence of these receptors (Figs. 2, 3). The transcriptional activities of WT and variant-type enhancer-promoters were not affected by pregnanediol, even in the presence of these receptors. UGT1A1 activity in early infancy is less than 1% of adult activity, which is reached by 3 mo of age (32). This change is believed to involve the induction of the UGT1A1 enzyme by increasing transcriptional activity of the *UGT1A1* gene. Our study suggests that pregnanediol does not inhibit the neonatal induction of UGT1A1.

Variant-type enhancer-promoter is the prevalent cause of Gilbert syndrome. In our previous study of BMJ in the Japanese population, the variation was not a risk factor for BMJ or neonatal hyperbilirubinemia (18,19). Bancroft *et al.* (33) also reported that the variant-type enhancer-promoter did not significantly affect neonatal hyperbilirubinemia. The variant-type enhancer-promoter might therefore be less effective in giving rise to BMJ than G71R. In the European population, however, this variant has been reported to be a risk factor for BMJ (34). This report is inconsistent with our observation that pregnanediol did not inhibit transcriptional activity of WT or of variant-type enhancer-promoter. It is possible that further unknown mutations, specific to Caucasians, link to the variant, and that ingredients of breast milk other than pregnanediol are involved in BMJ caused by the variation.

The recent report by Fujiwara *et al.* (35) with human *UGT1A1* gene complex, using humanized mice, revealed that bilirubin clearance during the neonatal period is associated not with hepatic UGT1A1 but with intestinal UGT1A1. Furthermore, A(TA)7TAA causes a decline in expression of hepatic UGT1A1, but the induction of UGT1A1 in the small intestine in the neonatal period is independent of the polymorphism in the mice. These findings support and explain the observation that A(TA)7TAA is not a risk factor of BMJ (18,19). Although the regulatory sequence responsible for intestinal induction of UGT1A1 has not yet been fully clarified, PXR activator, pregnenolone-16α-carbonitrile has reported to induce UGT1A1 in mouse small intestine (31). If PXR is responsible for inducing intestinal UGT1A1 in neonatal period, our results indicate that pregnanediol does not inhibit the induction.

We found that enzyme activity of WT UGT1A1 was not inhibited in the presence of pregnanediol but enzyme activity of G71R-UGT1A1 decreased (Fig. 4). The relative enzyme activity (G71R/WT) decreased to 51% ($p = 0.02$). G71R is a very common mutation of UGT1A1 in Gilbert syndrome in East Asians (14,18) and has been reported as a cause of BMJ on the infantile side by Maruo *et al.* (19). Pregnanediol in breast milk may directly inhibit UGT1A1 expressed in the human intestine in carriers of G71R (35). The present results, demonstrating the inhibitory effect of pregnanediol on G71R-UGT1A1, confirm previous results and clarify the underlying mechanism of BMJ (17).

In the neonatal period, UGT1A1 activity is very low (32). Furthermore, pregnanediol inhibits glucuronidation activity of G71R-UGT1A1, which has lower activity than WT UGT1A1. These observations may explain why severe unconjugated hyperbilirubinemia, with a serum bilirubin concentration above 20 mg/dL (340 μM), occasionally occurs in carriers of G71R. Beyond 4 mo of age, UGT1A1 activity runs at adult levels, as reported by Onishi *et al.* (32), and prolonged unconjugated hyperbilirubinemia in infants with G71R variant disappears even if breast milk feeding continues. It is possible that BMJ is caused by the combined effect of three factors: 1) a low amount of UGT1A1-protein in the neonatal period; 2) decreased enzyme activity by G71R; and 3) inhibition of G71R-UGT1A1 by pregnanediol. The inhibitory effect of pregnanediol is also consistent with our finding that G71R is a risk factor for neonatal hyperbilirubinemia (18). When infants with G71R ingest pregnanediol in breast milk in the early neonatal period, neonatal hyperbilirubinemia, and BMJ, may manifest or be intensified.

Breast milk contains a number of steroids, such as progesterone, cortisol, estrone, estradiol and estriol, and pregnanediol (36–38). Although these steroids are found in breast milk in much lower concentrations than pregnanediol (15 to 45 μg/100 mL) (39), they may still inhibit the activity of UGT1A1. For a deeper understanding of BMJ, especially in Caucasians, it would be helpful to examine whether these steroids contribute to the development of BMJ, in addition to pregnanediol.

This study has shown that a combination of G71R and pregnanediol in breast milk is a cause of BMJ in an East Asian population. Further research into the components of breast milk is necessary to settle the exact mechanism of BMJ.

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