Bilirubin Uridine Diphosphate-Glucuronosyltransferase Variation Is a Genetic Basis of Breast Milk Jaundice

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Objective To evaluate the role of bilirubin *UDP-glucuronosyltransferase family 1, polypeptide A1* (*UGT1A1*) gene variations on prolonged unconjugated hyperbilirubinemia associated with breast milk feeding (breast milk jaundice [BMJ]).

Study design *UGT1A1* gene allelic variation was analyzed in 170 Japanese infants with BMJ with polymerase chain reaction-direct sequencing, and their genotypes compared with serum bilirubin concentrations. In 62 of 170 infants, serum bilirubin concentration was followed after 4 months of life. Genotypes were examined in 55 infants without BMJ.

Results Of 170 infants with BMJ, 88 (51.8%) were homozygous *UGT1A1*6*. Serum bilirubin concentrations (21.8 ± 3.65 mg/dL) were significantly greater than in infants with other genotypes ($P < .0001$). The Gilbert UGT1A1*28 allele was not detected in infants with BMJ, except in an infant who was compound heterozygous with *UGT1A1*6*. At 4 months of age, serum bilirubin concentration improved to >1 mg/dL, except in 2 infants who were homozygous *UGT1A1*7*. Homozygous *UGT1A1*6* was not detected in the control group.

Conclusion One-half of the infants with BMJ were homozygous *UGT1A1*6* and exhibited a serum bilirubin concentration significantly greater than other genotypes. This finding indicates that *UGT1A1*6* is a major cause of BMJ in infants in East Asia. Previous finding have demonstrated that 5β-pregnane-3α,20β-diol present in breast milk inhibits p.G71R-UGT1A1 bilirubin glucuronidation activity. Thus, prolonged unconjugated hyperbilirubinemia may develop in infants with *UGT1A1*6* who are fed breast milk. *(J Pediatr 2014;165:36-41)*.

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Prolonged unconjugated hyperbilirubinemia associated with breast milk feeding (breast milk jaundice [BMJ]) is a phenomenon in infants fed with mother's breast milk.[1] This phenomenon is observed from the late neonatal period to 4 months of age. Hyperbilirubinemia decreases when breast milk is replaced with infant formula, and even if breast milk feeding continues, prolonged unconjugated hyperbilirubinemia improves over time. BMJ causes anxiety in infants, their parents, and pediatricians. In addition, kernicterus (bilirubin encephalopathy) is an occasional risk in severe unconjugated hyperbilirubinemia induced by BMJ.[2] Arias et al[1] and Newman and Gross[3] reported in 1963 that breast milk was a factor in the development of neonatal jaundice. Many substances in breast milk were subsequently suspected to cause BMJ, including pregnane-3α,20β-diol, nonesterified fatty acid, and β-glucuronidase.[4-6] However, a reliable causative agent of BMJ has not yet been conclusively elucidated.[7-9]

In a previous study of BMJ, we showed an association between BMJ and variants of the bilirubin UDP-glucuronosyltransferase (*UDP-glucuronosyltransferase family 1, polypeptide A1* [*UGT1A1*]) gene.[10] Our preliminary study on 17 infants with BMJ showed the c.211G>A (p.G71R) in the coding region of *UGT1A1* (*UGT1A1*6*) might cause BMJ. From 17 infants with BMJ, 8 with BMJ were homozygous for *UGT1A1*6*, 7 were heterozygous *UGT1A1*6*, and 2 infants did not express the *UGT1A1*6* allele.

UGT1A1 (EC 2.4.1.17) belongs to the UDP-glucuronosyltransferase type 1 (UGT1) family and plays a role in phase II drug metabolism.[11] UGT1A1 catalyzes glucuronidation of many endobiotics and xenobiotics, converting hydrophobic substances to hydrophilic substances as a detoxification.[12] Bilirubin is selectively catalyzed by UGT1A1.[13] Defects in the *UGT1A1* gene generate hereditary unconjugated hyperbilirubinemia, specifically Crigler-Najjar syndrome type I (MIM #21880), type II (MIM #606785), and Gilbert syndrome (MIM

BMJ	Breast milk jaundice
gtPBREM	Phenobarbital responsive enhancer module
PCR	Polymerase chain reaction
UGT1	UDP-glucuronosyltransferase type 1
UGT1A1	Bilirubin UDP-glucuronosyltransferase 1 family, polypeptide A1

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#143500).[13-17] Crigler-Najjar syndrome type I and type II are severe and moderate phenotypes of hereditary unconjugated hyperbilirubinemia, respectively, in which the hyperbilirubinemia is life long.[18,19] The clinical diagnosis of Gilbert syndrome occurs in approximately 3%-8.6% of the population.[20-22] Two frequent polymorphisms associated with Gilbert syndrome are a missense mutation in exon 1 c.211G>A generating a p.G71R change (*UGT1A1*6*) and a c.-3279T>G in the promoter region that is linked to the A(TA)7TAA in the TATA box (*UGT1A1*28*).[23]

Among white, black, and west Asian subjects, homozygous *UGT1A1*28* is associated with the clinical diagnosis of Gilbert syndrome.[16,17] However, in east Asian patients (Japanese, Koreans, and Chinese), *UGT1A1*6* is an important cause of adult hyperbilirubinemia, which is described clinically as Gilbert syndrome.[24] In this study, we demonstrate that during neonatal development, the role of the UGT1A1*6 allele predominates over the other polymorphisms in its contribution towards the onset of breast milk induced neonatal hyperbilirubinemia.[25] Thus, the *UGT1A1*6* allele is predicted to be a risk factor for breast milk–induced jaundice during neonatal development.

Methods

We studied 170 Japanese infants (95 male and 75 female) with prolonged unconjugated hyperbilirubinemia associated with breast milk feeding. Infants had a gestational age greater than 35 weeks (range, 35 weeks, 1 day to 41 weeks, 3 days; mean, 38 weeks, 5 days ± 10.6 days). Birth weights were greater than 2300 g (2305-3902 g; mean, 3003 ± 385 g). All infants showed apparent prolonged unconjugated hyperbilirubinemia beyond 3 weeks of life. Total and indirect bilirubin concentrations at diagnosis ranged from 7.3 mg/dL to 32.5 mg/dL (124.8-555.7 μmol/L), and more than 95% of bilirubin was indirect. Except for jaundice, the infants were healthy and did not show signs of hemolytic anemia, liver dysfunction, or hypothyroidism. Serum bilirubin concentrations were within the normal range (<1 mg/dL) or jaundice disappeared visually for all infants at 4 months of age even if breast milk feeding continued, except for infants with a particular genotype [homozygous p.Y486D (*UGT1A1*7*)].[26] We followed serum bilirubin concentrations after 4 month of age in 62 cases and checked the association between genotypes and final serum bilirubin concentrations. The project was approved by the ethics committee of Shiga University of Medical Science.

The control group comprised 55 term infants (21 male and 34 female). All infants were fed breast milk. At an obligatory neonatal health check at 1 month of age, they showed no visible evidence of prolonged jaundice. Mean gestational age was 39 weeks 4 days ± 7 days (range, 37 weeks, 4 days to 41 weeks), and birth weights were greater than 2300 g (mean, 3050 ± 337 g; range, 2386-3900 g). After informed consent from the parents was received, genomic DNA was extracted from lymphocytes in stored cord blood.

Sequence Analysis of *UGT1A1*

For sequence analysis of *UGT1A1* polymorphisms, genomic DNA was isolated from the leucocytes of infants, with parental informed consent. We amplified exons, the promoter region, and phenobarbital responsive enhancer module (gtPBREM) of UGT1A1 from genomic DNA by using polymerase chain reaction (PCR). In brief, approximately 100 ng of total genomic DNA was amplified with pairs of oligonucleotide primers. Exons 2, 3, and 4 and their intervening introns were simultaneously amplified as a single DNA fragment using a primer pair of 5′-CTCTATCT CAAACACGCATGCC-3′/ 5′-TTTTATCATGAATGCCATG ACC-3′. The 5′ region of UGT1A1, including the TATA box to exon 1, exon 5, and gtPBREM, was amplified separately with primer pairs of 5′-AAGTGAACTCCCTGC TACCTT-3′/5′-GCTTGCTCAGCATATATCTGGG-3′ (5′-region to exon 1), 5′-GAGGATTGTTCATACCACAGG-3′/ 5′-GCACTCTGGGGCTGATTAAT-3′ (exon 5), and 5′-CTGG GGATAAACATGGGATG-3′/5′-CACCACCACTTCTGGAAC CT-3′ (gtPBREM), respectively. Conditions for PCR were as follows: initial denaturation for 2 minutes at 94°C, followed by 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C for 30 cycles with a Minicycler (MJ Research, Inc, Watertown, Massachusetts). A final extension for 10 minutes at 72°C was performed to ensure complete extension of PCR products.

The sequences of the amplified DNA fragments were determined directly using the following sequencing primers. Sequence primers used for the determination of gtPBREM, TATA box, and coding region are as follows: for sequencing of gtPBREM: 5′-TGAGTTTATATAACCTC-3′; for the TATA box and exon 1: 5′-CTATTTCATGTCCCCTCTGC-3′, 5′-GT CTTTTGTTAGTCTCGGGC-3′, 5′-TTGTTGTGCAGTAAG TGGGA-3′, 5′-CCATTCTCCTACGTGCCCAG-3′, and 5′-AA GGGTTGCATACGGGGAATA-3′; for exon 2: 5′-GGAAGCT GGAAGTCTGGG-3′; for exon 3: 5′-CTAGTTAGTATAGCA GAT-3′; for exon 4: 5′-CAGCTGTGAAACTCAGAG-3′; and for exon 5: 5′-TGCTGACAGTGGCCTTCATC-3′ and 5′-GG TAGCCATAAGCACAACAT-3′. The sequences of the amplified DNA fragments were determined directly using a BigDye Terminator v1.1 Cycle Sequencing Kit and Genetic analyzer ABI Prism 3130xl (Applied Biosystems, Carlsbad, California).

Statistical Analyses

Serum bilirubin concentrations for the different genotypes detected were analyzed by ANOVA and the Scheffé test for pairwise comparisons using JMP9 (SAS Institute Inc, Cary, North Carolina) and Statview 4.5 (Abacus Corporation, Baltimore, Maryland). The analysis was performed among 5 frequently observed groups. Homozygous *UGT1A1*6* (encodes the p.G71R variant), compound heterozygous for *UGT1A1*6* and *UGT1A1*60* (promoter c.-3279T>G in the gtPBREM region), heterozygous *UGT1A1*6*, and homozygous *UGT1A1*1* (the normal common allele). All DNA samples were screened for mutations in the gtPBREM region, promoter and TATA box, exons, and exon-intron boundaries of the *UGT1A1* gene.