RESEARCH ARTICLE

Multiple Genetic Modifiers of Bilirubin Metabolism Involvement in Significant Neonatal Hyperbilirubinemia in Patients of Chinese Descent

Hui Yang1,2, Qian Wang1*, Lei Zheng1, Min Lin2, Xiang-bin Zheng2, Fen Lin2, Li-Ye Yang2*

1 Laboratory Medical Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province, P. R. China, 2 Central Laboratory, Chaozhou Central Hospital Affiliated to Southern Medical University, Chaozhou, Guangdong Province, P. R. China

☯ These authors contributed equally to this work.
* nfky_well@163.com (QW); yangleeyee@sina.com (LY)

Abstract

The potential for genetic variation to modulate neonatal hyperbilirubinemia risk is increasingly being recognized. A case-control study was designed to assess comprehensive contributions of the multiple genetic modifiers of bilirubin metabolism on significant neonatal hyperbilirubinemia in Chinese descendents. Eleven common mutations and polymorphisms across five bilirubin metabolism genes, namely those encoding UGT1A1, HMOX1, BLVRA, SLCO1B1 and SLCO1B3, were determined using the high resolution melt (HRM) assay or PCR-capillary electrophoresis analysis. A total of 129 hyperbilirubinemic infants and 108 control subjects were evaluated. Breastfeeding and the presence of the minor A allele of rs4148323 (UGTA*6) were correlated with an increased risk of hyperbilirubinemia (OR=2.17, P=0.02 for breastfeeding; OR=9.776, P=0.000 for UGTA*6 homozygote; OR=3.151, P=0.000 for UGTA*6 heterozygote); whereas, increasing gestational age and the presence of –TA7 repeat variant of UGT1A1 decreased the risk (OR=0.721, P=0.003 for gestational age; OR=0.313, P=0.002 for heterozygote TA6/TA7). In addition, the SLCO1B1 and SLCO1B3 polymorphisms also contributed to an increased risk of hyperbilirubinemia. This detailed analysis revealed the impact of multiple genetic modifiers on neonatal hyperbilirubinemia. This may support the use of genetic tests for clinical risk assessment. Furthermore, the established HRM assay can serve as an effective method for large-scale investigation.

Introduction

Neonatal jaundice or hyperbilirubinemia frequently manifests as a pediatric complex trait or disorder, which is still prevalent (1%) in the newborn population today [1, 2]. Both genetic and
environmental factors contribute to the development of neonatal hyperbilirubinemia, and the importance of genetic contributions in this disorder has been recently recognized [3–5].

The main feature of neonatal hyperbilirubinemia is an increased bilirubin production that cannot be matched by glucuronidation and the elimination of bilirubin [6]. Growing literature has shown that genetic variations across multiple bilirubin metabolism genes might affect serum bilirubin levels in healthy adult populations of different ethnic backgrounds [7–11]. However, the role of the genetic modifiers of bilirubin metabolism on neonatal hyperbilirubinemia has not yet been conclusively elucidated.

To explore the complex role of multiple genetic modifiers on unconjugated neonatal hyperbilirubinemia, eleven common polymorphisms across five bilirubin metabolism genes [Heme oxygenase-1 (HMOX1), biliverdin reductase A (BLVRA), hepatic bilirubin-conjugating isoenzyme uridinediphosphoglucuronosyltransferase 1A1 (UGT1A1), and solute carrier organic anion transporter family member 1B1 (SLCO1B1) and 1B3 (SLCO1B3)] were determined in our case-control study of Chinese neonates. In addition, we developed a rapid genotype screening assay for a comprehensive analysis of the nine single base polymorphisms of the eleven selected SNPs using PCR and high-resolution melt analysis (HRM) technology. All selected variants, along with clinical parameters (sex, age and feeding method), were included in our associated evaluation. The data obtained in this study may broaden our knowledge on the molecular pathogenesis of neonatal hyperbilirubinemia and provide genetic markers for clinical risk assessment.

Materials and Methods

Study population and sample collection

This was a retrospective case-control study conducted in the pediatric center of a single hospital (Chaozhou Central Hospital affiliated to Southern Medical College). Peripheral blood samples were prospectively collected from newborns consecutively admitted to the study center from November 2011 to September 2014. Clinical records including the birth date, gender, birth weight, delivery method, gestational age, feeding method, total serum bilirubin levels (TSB) and peak bilirubin levels before phototherapy were reviewed. Eligible infants were term infants with a gestational age of more than 37 weeks, a birth weight >2500 g and no major birth abnormalities and serious illness.

Hyperbilirubinemia was diagnosed and treated according to the updated clinical guidelines of the Chinese Medical Association for neonates [12]. The recorded peak TSB was used to divide the study subjects into case and control subjects. The case subjects included jaundiced infants with a maximum TSB that required phototherapy based on the above guidelines. Neonates with known clinical risk factors for developing neonatal hyperbilirubinemia, such as hemolysis (a positive Coombs’ test), glucose-6-phosphate dehydrogenase deficiency, cephalohematoma, infection, perinatal asphyxia, and major organ abnormality, were excluded. Control subjects were term neonates, admitted to the study center for other reasons than jaundice during the same period, with a TSB not requiring phototherapy according to the same guidelines.

The study was approved by Ethics Committee of Chaozhou Central Hospital. Because the data were analyzed anonymously and blood samples for this study were used after the completion of clinical diagnostic work (blood routine examination), the hospital ethics committees approved a waiver of written consent.

Molecular Analysis

The genomic DNA was extracted from surplus EDTA anti-coagulated whole blood samples using a DNA mini-preparation kit (Decipher Bioscience Shenzhen Ltd., Shenzhen, China).
100uL anti-coagulated whole blood was used for each DNA specimen extraction. The specific mutations of UGT1A1, HO-1, BLVRA, SLCO1B1, and SLCO1B3 were analyzed as described below. The information on the eleven polymorphisms was listed in S1 Table.

The (GT)ₙ repeat variations in the HMOX1 (dbSNP rs1805173) and the (TA)ₙ repeat variations in the UGT1A1(dbSNP rs81753472) gene promoters were determined by fragment (size-based) analysis. Both promoter regions were separately amplified by polymerase chain reaction (PCR) with a FAM-labeled sense primer. The PCR products were mixed with the Gene Scan-400 size standard (size range: 50–400 bp, Applied Biosystems Inc.), and analyzed on the ABI 3700 Genetic Analyzer (Applied BioSystems, Foster City, California) using the software GeneMapper 4.0 (Applied BioSystems). To confirm the sizes of the (GT)ₙ and (TA)ₙ repeats, selected samples were subjected to sequence analysis on an ABI Genetic Analyzer. The sequence results were used to confirm the molecular weight as determined by fluorescence labeling.

The polymorphisms rs4148323, rs35390960, rs6742078 and rs1018124 from the UGT1A1 gene, rs699512 from the BLVRA gene, rs2306283 and rs4149056 from the SLCO1B1 gene, and rs2417940 and rs2117032 from the SLCO1B3 gene were identified by PCR and HRM analysis using Lightcycler instruments. The reaction conditions were 95°C for 3 min, followed by 10 cycles of 98°C for 10 s, 65°C for 5 s, and 72°C for 15 s, and 50 cycles of 98°C for 10 s, 55°C for 5 s, and 72°C for 15 s, with final extension at 72°C for 90 s. The cycling conditions were the same for all amplicons. After amplification, HRM analysis was performed using the software LightCycler 480 SW 1.5 (Roche Diagnostics) as described previously [13, 14]. Each DNA sample was amplified twice to validate the coherence of the curve shapes. Selected samples were amplified and directly sequenced to verify the accurate genotype of the HRM assay. Primers for the HRM assay were given in S1 Table.

Statistical analysis

χ²-test, Fisher’s exact test, Student’s t-test, and the Mann-Whitney nonparametric test were used to compare the differences in the clinical parameters, as appropriate. The Hardy-Weinberg equilibrium (HWE) test for the (GT)ₙ repeat of HMOX1 was performed using the exact test [15]. The HWE test for the other loci was examined using a chi-square test. Linkage disequilibrium (LD) between the 4 polymorphisms within UGT1A1 was calculated, and the analysis of inferred haplotypes was also performed using the web tool SNPSstats [16] (http://bioinfo.iconcologia.net/SNPSstats). Furthermore, the best model of inheritance for each SNP (co-dominant, dominant, recessive, overdominant or additive) was selected based on the lowest Akaike information criterion.

Logistic regression models were performed to evaluate the association between the specific polymorphism or haplotypes and the development (case vs. control) and severity of neonatal hyperbilirubinemia (severe, mild/moderate jaundice or no jaundice). Initially, the estimated odds ratio (OR) and corresponding 95% confidence interval (CI) were measured between the case and control groups using only binary logistic regression after adjusting for known clinical risk factors for neonatal hyperbilirubinemia, including sex, breastfeeding, and age. Then, a multivariate ordinal logistic regression model was used to evaluate the association of the gene variations and clinical parameters with the severity of hyperbilirubinemia categorized by the peak TSB levels recorded before phototherapy. The threshold for this categorization was taken from the recommendations of the 2003 National Institute of Child Health and Human Development (NIHCD), National Institutes of Health, United States of America conference [1], including serious hyperbilirubinemia (TSB levels ≥20 mg/dL), mild/moderate jaundice (TSB levels ≥12 mg/dL) and no jaundice. The stepwise selection procedure was used to investigate the most significant predictors of hyperbilirubinemia. Variables with P < 0.10 from the
separated binary logistic analysis were included in the stepwise selection. Fitting model in the ordinal regression was evaluated by the parallel line test and goodness of fitting test. Both the non-significant P-value (p > 0.05) of the parallel line test and goodness of fit test indicated that the ordered models were appropriate [17]. In all the regression analyses, the common homozygote genotype in the control population was considered as the reference category. P < 0.1 was considered to be significant. Subset analysis was further carried out to evaluate for a potential synergistic effect between genetic modifiers and covariates, including the sex, breastfeeding status and gestational age of the included neonates.

All statistical analysis was performed using SPSS version 16 for windows (SPSS, Chicago, Illinois, USA) and SNPstat Software. All statistical tests were 2-sided, and statistical significance was set at P < 0.05.

Results
Clinical characteristics
Eighty-one jaundiced neonates were excluded based on the criteria for exclusion as described previously, and a total of 129 term newborns with significant hyperbilirubinemia, including 42 neonates with peak TSB > 20 mg/dL (342 μmol/L), were enrolled as case subjects. All the neonates in the case group received phototherapy except 2 neonates with prolonged unconjugated hyperbilirubinemia. The control subjects were 108 term neonates with no clinical jaundice [median TSB: 97.11 μmol/L (13.1–225.4 μmol/L)]. The clinical features of the subjects were summarized in Table 1. There were no statistically significant differences between case subjects and control subjects with respect to birth weight and sex. Although late preterm neonates were excluded, the gestational age still differed between the two groups with 39.0 ± 1.3 wk vs. 39.6 ± 1.1 wk (P < 0.001). Furthermore, the case neonates were breastfed more often than the control neonates.

| Table 1. Demographic and clinical features of the neonates in the case and control groups. |
|---------------------------------------------------------------|
|                                                               | Case | Control | p     |
| Sex (n)                                                        |      |         |       |
| Male                                                           | 80   | 64      | 0.707 |
| Female                                                        | 49   | 44      |       |
| Gestational age (week)                                        | 39.0±1.31 | 39.5±1.20 | 0.003 |
| Birth weight (kg)                                             | 3.15±0.38 | 3.16±0.37 | 0.771 |
| Maximum TSB levels (μmol/L)                                   | 312.48(250.5–564.8) | 97.11(13.1–225.4) | 0.048 |
| Feeding                                                       |      |         |       |
| Breastfeeding                                                 | 59   | 41      |       |
| Breast and formula                                            | 40   | 28      |       |
| Formula                                                       | 24   | 31      |       |
| Unknown                                                       | 6    | 8       | 0.370 |
| Birth delivery                                                |      |         | 0.104 |
| Vaginal                                                       | 64   | 65      |       |
| Cesarean                                                      | 65   | 43      |       |

a Mean±SD.  
b Median (95% CI).  
doi:10.1371/journal.pone.0132034.t001
Genotype results
HRM analysis was applied for the rapid genotyping of the 9 selected common single base variants in our study cohort. As shown in Fig 1, a heterozygous mutation could be easily distinguished from wild-type samples based on differences in the HRM curve shape. The HRM curve shapes for the homozygous mutations were similar to those of the wild-type subjects, and the homozygous mutations were detected by a modified HRM analytical strategy as described in our previous study [13]. Each SNP for the 50 samples was selected for direct sequencing. All the test samples were accurately genotyped as confirmed by the sequencing results. Therefore, we believed that HRM could be used as a general and rapid method for large-scale clinical investigation.

The minor allele frequencies (MAFs) of the polymorphisms in our study cohort were presented in Table 2. The MAF of SNP rs3539046 in UGT1A1 was lower than 0.003 and was excluded from further analysis. None of the polymorphisms showed statistically significant deviations from the HWE in our control subjects except rs4149056, which was excluded from the sub-analysis. Strong pairwise LD was observed between the five polymorphisms (each |D'|>0.8) within UGT1A1, whereas a low pairwise LD was present between the polymorphisms within the SLCOs family (SLCO1B1, SLCO1B3). The LD pattern across the multiple SNPs of UGT1A1 was shown in S2 Table. A high r² value (0.885) was present only between the (TA)ₙ repeat polymorphism and the rs6742078 of UGT1A1 gene.

The number of (GT)ₙ repeats in the HO-1 gene promoter region in this study population ranged from 15 to 40, with one peak at 23 GT repeats and the other peaks at close to 30 GT repeats. According to previous report from the HO-1 association and functional studies [18, 19], we divided the allelic repeats into three subgroups: short alleles (S: <24 GT), middle alleles (M: 24–29 GT), and long alleles (L: >29 GT). We then further classified the 6 genotypes into 2 groups as was done in previous studies [7, 20]. Subjects carrying the S/S or S/M GT repeats were classified as Group 1, whereas subjects carrying M/M, M/L, or L/L GT repeats were Group 2.

Association study
Association analysis of the 10 common variants with hyperbilirubinemia was first evaluated using separate binary logistic regressions (SNPstats software). The allele, genotype and haplotype of the three SNPs in UGT1A1, including rs4148323, rs6742078 and (TA)ₙ, were found to have considerable differences between cases and controls. There was also a strong association with neonatal hyperbilirubinemia after being adjusted for known clinical risk factors for neonatal hyperbilirubinemia, including sex, breastfeeding, and age (Tables 2 and 3). Specifically, neonates harboring minor alleles of rs4148323 (known as UGT1A1*6, 211G>A) were found to have a significantly increased risk of hyperbilirubinemia (OR_adj = 13.02; p = 1e-04 for UGT1A1*6 homozygote, OR_adj = 2.69; p = 1e-04 for UGT1A1*6 heterozygote), whereas minor alleles at rs6742078 and (TA)ₙ[rs8175347] were observed to have a protective effect on the risk of hyperbilirubinemia (OR_adj = 0.16; p = 0.0001 for rs6742078; OR_adj = 0.25; p = 0.000 for (TA)ₙ). Similarly, haplotype analysis showed that ATA(TA)ₙ (rs4148323-rs6742078-rs108124-(TA)ₙ) increased the risk (OR_adj = 3.00; p = 4e-04), whereas GAT(TA)ₙ produced a protective effect for hyperbilirubinemia (OR_adj = 0.22; p = 0.0018) compared to the most common haplotype GTA(TA)ₙ. Two SNPs in the SLCOs family also showed a trend towards an increased risk of hyperbilirubinemia in the univariable regression analysis after adjusting for potential covariates, including the age, gender, and feeding method (OR_adj = 2.16; p = 0.098 for rs2306283 in recessive model; OR_adj = 1.61; p = 0.096 for rs2117032 in overdominant model).

The neonates were further stratified into three groups by the peak TSB levels recorded for each neonate with severe (TSB levels ≥20 mg/dL) or mild/moderate hyperbilirubinemia (TSB
Fig 1. HRM analysis of 9 single base polymorphisms across the UGT1A1, SLCO1B1, SLCO1B3 and BLVRA genes. (a): rs4148323 G>A; (b): rs6742078 T>G; (c): rs35390940 C>A; (d): rs108124 A>G; (e): rs2306283 G>A; (f): rs4149056 T>C; (g): rs2117032 T>C; (h): rs2417940 T>C; (i): rs699512 A>G.

doi:10.1371/journal.pone.0132034.g001
Table 2. Minor allelic, genotypic, and haplotype distributions of the 11 polymorphisms in the bilirubin metabolism gene: control vs. case groups.

| polymorphism         | location | control    | case        | $P_{H-W}$ | $P_{\text{allele}}$ | $P_{\text{genotype}}$ |
|----------------------|----------|------------|-------------|-----------|---------------------|------------------------|
| HO-1$^{a}$           | (GT)$_n$ |            |             |           |                     |                        |
| S allele             | promoter |            |             | 0.34      | 0.38                | 0.59                   |
| M allele             |          |            |             | 54 (25.2%)| 80 (31.0%)          |                        |
| L allele             |          |            |             | 56 (26.2%)| 63 (24.4%)          |                        |
| SS                   |          |            |             | 104 (48.6%)| 115 (44.6%)        |                        |
| SM                   |          |            |             | 7 (6.5%)  | 13 (10.1%)          |                        |
| SL                   |          |            |             | 10 (9.3%) | 17 (13.2%)          |                        |
| MM                   |          |            |             | 30 (28.0%)| 37 (28.7%)          |                        |
| ML                   |          |            |             | 32 (29.9%)| 38 (29.5)          |                        |
| LL                   |          |            |             | 21 (19.6%)| 20 (15.5%)          |                        |
| UGT1A1               | (TA)$_n$ |            |             |           |                     |                        |
| TA$_T$               | Exon 1   |            |             | 0.052     | 0.0002              | 8.38e-05               |
| TA$_G$               |          |            |             | 34 (15.7%)| 14 (5.4%)          |                        |
| TA$_T$/TA$_T$        |          |            |             | 182 (84.3%)| 244 (94.6%)       |                        |
| TA$_G$/TA$_T$        |          |            |             | 0 (0.00%) | 0 (0.00%)          |                        |
| TA$_G$/TA$_G$        |          |            |             | 34 (31.5%)| 14 (10.9%)         |                        |
| TA$_G$/TA$_G$        |          |            |             | 74 (68.5%)| 115 (81.9%)        |                        |
| rs4148323            | Exon 1   |            |             |           |                     |                        |
| A allele             |          |            |             | 25 (11.7%)| 74 (28.7%)         | 0.666                  |
| A/A                  |          |            |             | 1 (0.9%)  | 12 (9.3%)          |                        |
| G/A                  |          |            |             | 23 (21.5%)| 50 (38.8%)         |                        |
| G/G                  |          |            |             | 83 (77.6%)| 67 (51.9%)         |                        |
| rs35390960           | Exon 1   |            |             |           |                     |                        |
| A allele             |          |            |             | 5 (2.7%)  | 2 (0.8%)           | 0.78                   |
| A/A                  |          |            |             | 1 (0.9%)  | 12 (9.3%)          | 0.13                   |
| C/A                  |          |            |             | 5 (5.5%)  | 2 (1.7%)           |                        |
| C/C                  |          |            |             | 86 (94.5%)| 116 (98.3%)        | 0.13                   |
| rs6742078            | Intron 1 |            |             |           |                     |                        |
| G allele             |          |            |             | 33 (15.4%)| 9 (3.9%)          | 0.059                  |
| G/G                  |          |            |             | 0 (0.00%) | 0 (0.00%)          | 1.64e-05              |
| T/G                  |          |            |             | 33 (30.8%)| 9 (7.8%)          | 5.52e-06              |
| T/T                  |          |            |             | 75 (69.2%)| 117 (92.2%)        |                        |
| rs108124             | Intron 1 |            |             |           |                     |                        |
| G allele             |          |            |             | 36 (18.4%)| 53 (22.3%)         | 0.37                   |
| G/G                  |          |            |             | 2 (2.0%)  | 3 (2.5%)           | 0.31                   |
| A/G                  |          |            |             | 32 (32.7%)| 47 (39.5%)         | 0.54                   |
| A/A                  |          |            |             | 64 (65.3%)| 69 (58%)           |                        |
| SLCO1B1              | Exon 5   |            |             |           |                     |                        |
| rs2306283            | Exon 5   |            |             |           |                     |                        |
| A allele             |          |            |             | 49 (22.7%)| 75 (29.1%)         | 0.428                  |
| A/A                  |          |            |             | 7 (6.5%)  | 17 (13.2%)         | 0.11                   |
| G/A                  |          |            |             | 35 (32.4%)| 41 (31.8%)         | 0.22                   |
| A/A                  |          |            |             | 66 (61.1%)| 71 (55.0%)         |                        |
| rs4149056            | Exon 6   |            |             |           |                     |                        |
| C allele             |          |            |             | 39 (18.2%)| 33 (12.8%)         | 0.003                  |
| C/C                  |          |            |             | 8 (7.5%)  | 2 (1.6%)           |                        |

(Continued)
In the multivariate ordinal regression analysis, the following 4 SNPs, together with 2 demographic predictors were significant and remained in the model. The estimated OR and 95%CI of the six predictors of hyperbilirubinemia were shown in Table 4. Neonates of older gestational age and TA7 repeat variants of UGT1A1 (UGTA/C328) decreased the risk of hyperbilirubinemia (OR = 0.721, 95%CI: 0.583–0.895, P = 0.003 for gestation age; OR = 0.313, 95%CI: 0.148–0.660, P = 0.002 for heterozygote TA6/TA7), whereas breastfeeding and presence of minor A allele of rs4148323 (UGTA/C36) increased the hyperbilirubinemia risk (OR = 2.17, 95%CI: 1.127–4.203, P = 0.02 for breastfeeding; OR = 9.776, 95%CI: 2.812–34.02, P = 0.000 for UGTA*6 homozygote; OR = 3.151, 95%CI: 1.729–5.748, P = 0.000 for UGTA*6 heterozygote).

In addition, the homozygote A/A of rs2306283 in SLCO1B1 and the heterozygote C/T of rs2117032 in SLCO1B3 also contributed to an increased risk of hyperbilirubinemia (ORadj = 2.401, 1.037–5.556, p = 0.041 for rs2306283 in the recessive model; ORadj = 2.10, 1.198–3.684, p = 0.01 for rs2117032 in the overdominant model).

### Table 2. (Continued)

| polymorphism | location | control | case | PH-W | P allele | P genotype |
|--------------|----------|---------|------|-------|----------|------------|
| T/C          | 23 (21.5%) | 29 (22.5%) |      |       |          |            |
| T/T          | 76 (71.0%) | 98 (76.0%) |      |       |          |            |
| SLCO1B3      |          |         |      |       |          |            |
| rs2117032    | 3’-UTR   | 0.50    | 0.98 | 0.23  |          |            |
| C allele     | 101 (48.1%) | 121 (48.0%) |      |       |          |            |
| C/C          | 26 (24.8%) | 24 (19.0%) |      |       |          |            |
| T/C          | 49 (46.7%) | 73 (57.9%) |      |       |          |            |
| T/T          | 30 (28.6%) | 29 (23.0%) |      |       |          |            |
| rs2417940    | Intron 7  | 0.65    | 0.93 | 0.91  |          |            |
| T allele     | 37 (18.0%) | 46 (18.3%) |      |       |          |            |
| T/T          | 4 (3.9%) | 4 (3.2%) |      |       |          |            |
| C/T          | 29 (28.2%) | 38 (30.2%) |      |       |          |            |
| C/C          | 71 (68%) | 84 (66.7%) |      |       |          |            |
| BLVRA        |          |         |      |       |          |            |
| rs699512     | Exon 2   | 0.48    | 0.46 | 0.93  |          |            |
| G            | 56 (26.4%) | 76 (29.5%) |      |       |          |            |
| G/G          | 6 (5.7%) | 11 (8.5%) |      |       |          |            |
| A/G          | 44 (41.5%) | 54 (41.9%) |      |       |          |            |
| A/A          | 56 (52.6%) | 64 (49.6%) |      |       |          |            |
| Haplotypeb   |          |         |      |       |          |            |
| ATA6         | 72 (30.5%) | 22 (11.7%) |      | 3.64e-06 |          |            |
| GTG6         | 53 (22.4%) | 34 (17.4) |      | 0.21   |          |            |
| GGA7         | 9 (3.8%) | 31 (16.1%) |      | 1.21e-05 |          |            |
| GTA7         | 4 (1.7%) | 1 (0.5%) |      | 0.34   |          |            |
| GTA6         | 98 (41.6%) | 103 (53.4%) |      | 0.012  |          |            |
| Others       | -        | -       |      |        |          |            |

a S<23(GT); 23M<29(GT); L>29(GT).
b other haplotypes had frequencies less than 1%.
c Hardy-Weinberg Equilibrium test p value.

doi:10.1371/journal.pone.0132034.t002

levels ≥12 mg/dL) or no jaundice (as recommended by 2003 NIHCD conference) [1]. In the multivariate ordinal regression analysis, the following 4 SNPs, together with 2 demographic predictors were significant and remained in the model. The estimated OR and 95%CI of the six predictors of hyperbilirubinemia were shown in Table 4. Neonates of older gestational age and TA7 repeat variants of UGT1A1 (UGTA*28) decreased the risk of hyperbilirubinemia (OR = 0.721, 95%CI: 0.583–0.895, P = 0.003 for gestation age; OR = 0.313, 95%CI: 0.148–0.660, P = 0.002 for heterozygote TA6/TA7), whereas breastfeeding and presence of minor A allele of rs4148323 (UGTA*6) increased the hyperbilirubinemia risk (OR = 2.17, 95%CI: 1.127–4.203, P = 0.02 for breastfeeding; OR = 9.776, 95%CI: 2.812–34.02, P = 0.000 for UGTA*6 homozygote; OR = 3.151, 95%CI: 1.729–5.748, P = 0.000 for UGTA*6 heterozygote).

In addition, the homozygote A/A of rs2306283 in SLCO1B1 and the heterozygote C/T of rs2117032 in SLCO1B3 also contributed to an increased risk of hyperbilirubinemia (ORadj = 2.401, 1.037–5.556, p = 0.041 for rs2306283 in the recessive model; ORadj = 2.10, 1.198–3.684, p = 0.01 for rs2117032 in the overdominant model).
Table 3. Association analysis of the 10 polymorphisms in bilirubin metabolism genes and the risk of hyperbilirubinemia under different inheritance models: binary logistic regression.

| Gene  | Polymorphism | ORcrude (95%CI) | Pcrude  | ORadja (95%CI) | Padja  |
|-------|--------------|-----------------|---------|----------------|--------|
| Ho-1  | (GT)1n       | 1.60 (0.83–3.10) | 0.16    | 1.70 (0.83–3.48) | 0.14   |
|       | SS+SM        | reference       |         |                |        |
| SL+MM+ML+LL | reference |              |         |                |        |
| UGT1A1| (TA)1n       | 0.26 (0.13–0.53) | 1e-04   | 0.25 (0.12–0.50) | 1e-04  |
|       | TA6/TA7      | reference       |         |                |        |
| rs4148323|            |                |         |                |        |
|       | A/A          | A/G+G/G        | 1.36 (0.78–2.37) | 0.27 | 1.41 (0.78–2.56) | 0.25 |
|       | G/G          | reference       |         |                |        |
|       | SLCO1B1      | 1.55 (0.55–4.35) | 0.39    | 1.99 (0.63–6.25) | 0.23   |
|       | rs2306283    | A/A+G/G        |         |                |        |
|       | A/G+G/G      | 2.19 (0.87–5.50) | 0.083   | 2.16 (0.84–5.53) | 0.098  |
|       | A/A          | reference       |         |                |        |
|       | SLCO1B3      | 1.57 (0.93–2.65) | 0.087  | 1.61 (0.92–2.82) | 0.096  |
|       | rs2117032    | T/C+T/C        |         |                |        |
|       | T/C          | reference       |         |                |        |
|       | rs2417940    | 1.10 (0.62–1.96) | 0.74   | 1.06 (0.58–1.94) | 0.84   |
|       | C/C+T/T      | reference       |         |                |        |
| Haplotype b (Frequency) |  |  |  |  |  |
| ATA6  | (20.64%)     | 3.17 (1.80–5.58) | 1e-04   | 3.00 (1.66–5.43) | 4e-04  |
| GGTG6 | (20.63%)     | 1.42 (0.78–2.58) | 0.25    | 1.36 (0.73–2.53) | 0.33   |
| GGAT7 | (9.07%)      | 0.26 (0.12–0.60) | 0.0018 | 0.22 (0.09–0.52) | 7e-04  |
| GTA7  | (1.05%)      | 4.24 (0.46–39.46) | 0.21  | 6.67 (0.67–66.00) | 0.11   |
| GTA6  | (48.28%)     | reference       |         |                |        |
| Others c (0.31%) |            |                |         |                |        |

a Adjusted for age, gender, and feeding practice.

b Polymorphisms are in order of: rs4148323-rs6742078-rs108124-(TA)n.

c Other haplotypes had frequencies of less than 1%.

doi:10.1371/journal.pone.0132034.t003
In the subgroup analysis, the neonates were stratified by age, sex and feeding method, and we found that there existed an additive effect of the UGTA/C36 variant (211 G > A, Gly71Arg) and breastfeeding on the hyperbilirubinemia risk. As shown in Table 5, after adjusting for the sex and gestational age, the neonates carrying heterozygous and homozygous UGTA/C36 variants (G/A and A/A genotype) had a substantially higher risk of hyperbilirubinemia than those with the wild phenotype (G/G genotype) in the BF group (breastfed and mixed breastfed) rather than in the SF group (exclusively supplement formula-fed). Neonates with the homozygous UGTA/C36 variant that were exclusively fed with breast milk had the highest relative risk of hyperbilirubinemia development ($P = 0.018$).

**Discussion**

The pathogenesis of significant neonatal hyperbilirubinemia is often multifactorial, involving bilirubin overproduction, reduced conjugation and increased enterohepatic recycling [6, 21]. The purpose of the present study was to elucidate the comprehensive contributions of the multiple genetic modifiers of bilirubin metabolism on the development of significant hyperbilirubinemia in newborns of Chinese descent. Our results revealed that two independent genetic variants in the promoter and coding region of UGT1A1 genes had a substantial impact on the risk of neonatal hyperbilirubinemia. In addition, two polymorphisms in the SLCO family were
also associated with hyperbiliruinemia risk in our study cohort. Finally, our data demonstrated that there was a significant gene-environment interaction between the UGT1A1 gene coding region variation and breastfeeding.

The UGT1A1 coding sequence variant rs4148323 (known as UGT1A1*6, G211A), the most common cause of Gilbert syndrome in east Asians, was well documented and predominantly associated with TSB levels and neonatal hyperbilirubinemia risk in the Asian population [22–24]. Our results confirmed the strong association of UGT1A1*6 with the incidence and severity of hyperbilirubinemia. Furthermore, we observed a significant gene-environment interaction between UGT1A1*6 and breastfeeding. Recently, Chou et al. in Taiwan found that UGT1A1*6 was significantly associated with hyperbilirubinemia in exclusively breastfed neonates. In the current study, we further demonstrated that UGT1A1*6 was also a risk factor in mixed breastfed neonates. Moreover, we clearly demonstrated that UGT1A1*6 was not a significant risk predictor in neonates who were exclusively formula-fed. This was consistent with Chou’s observations [25], but was somewhat inconsistent with the results of a similar study in Taiwanese neonates by Huang et al. [22], who showed that UGT1A1*6 was also a risk factor of neonatal hyperbilirubinemia in neonates who were not fed with breast milk. The discrepancy between these findings may be due to differences in the categorization of the study populations. The supplement formula-fed group in our study included those neonates who were exclusively formula-fed. In contrast, the neonates in Chou and in Huang’s study received both formula and breast milk.

Several mechanisms have been proposed to explain the additive role of UGT1A1 gene variations and breastfeeding in significant neonatal hyperbilirubinemia. It has been demonstrated that pregnane-3(a), 20(b)-dol in breast milk inhibited bilirubin conjugation in the presence of the UGT1A1*6 polymorphic mutation of UGT1A1 [26]. Furthermore, later studies using the humanized UGT1 mouse model have clarified that breast milk reduced the expression of intestinal UGT1A1, which enhanced the risk of hyperbilirubinemia because UGT1A1 expression in the small intestine played an important role in bilirubin glucuronidation during the neonatal period [27, 28]. Although the UGT1A1*6 genotype was not evaluated in the UGT1A1*1 and UGT1A1*28 mouse models, these mechanisms explained the additive role of UGT1A1 gene variations and breastfeeding in significant neonatal hyperbilirubinemia.

The (TA)n repeat variant in the UGT1A1 promoter was another extensively studied variant. Previous epidemiological studies based on independent samples or GWAs samples from European and Asia adult populations have demonstrated that the long repeat of (TA)n was associated

### Table 5. Subgroup analysis of rs4148323 polymorphisms and the risk of hyperbilirubinemia depending on the feeding type.

| Feeding type                  | rs4148323 | rs4148323 |
|------------------------------|-----------|-----------|
|                              | Wildtype  | G/A       | A/A       | G/A+A/A   |
| Control (n)                  | Case (n)  | ORadj* (95%CI) |
| Formula-fed 19               | 18        | reference                              |
| Control (n)                  | Case (n)  | ORadj* (95%CI) |
| Breast and formula 23        | 21        | 0.95(0.39–2.32) |
| Control (n)                  | Case (n)  | ORadj* (95%CI) |
| Breastfed 34                 | 27        | 0.76(0.33–1.75) |

*Adjusted for sex and gestational age.
# Subgroups with P<0.05 are shown in bold.

doi:10.1371/journal.pone.0132034.t005
with increased TSB levels [7, 8, 10, 11, 29]. However, the role of this variant on neonatal hyperbilirubinemia risk was undefined and conflicting. For instance, homozygous A(TA)$_7$TAA variations in the promoter region of the UGT1A1 gene was found to be associated with neonatal hyperbilirubinemia in Caucasian, whereas most studies in east Asian countries failed to find this association [30]. Interestingly, two recent case-control studies in Chinese and Japanese breastfed neonates observed that the heterozygous (TA)$_7$ mutation decreased the risk of hyperbilirubinemia significantly (OR: 0.37; 95%CI: 0.15–0.89; p = 0.027) [20, 31]. In the present study, we also found an inverse association between the TA$_7$ repeat variant and the hyperbilirubinemia risk. Taken together, the TA$_7$ repeat variant of UGT1A1 (UGTA*28) seems to have a protective effect on hyperbilirubinemia development in Asia neonates. Although this conclusion contradicted the results of the traditional function study, Zhou et al further found higher bilirubin levels in neonates heterozygous for (TA)$_7$/(TA)$_6$ than in those homozygous for (TA)$_7$/(TA)$_7$ [32]. One study in Taiwanese neonates also reported a dose-dependent effect of UGTA*28 on the lower TSB levels (homozygote< heterozygote< wild type) [25]. Another study in jaundiced children also observed this trend, although the ethnicity of the subjects was not described [33]. It was unusual that the promoter variant showed different, even opposite, effects on TSB. The first suggested mechanism for this phenomenon come from Beutler et al. [34], who suggested that the (TA)$_n$ repeat might be a balanced polymorphism evolutionarily selected to maintain serum bilirubin in an optimal range in the face of largely undefined genetic and environmental pressures. Further studies are certainly needed to confirm this hypothesis.

Another SNP (rs6742078) in the UGT1A1 gene also showed a protective effect for neonatal hyperbilirubinemia. This SNP was reported to be highly linked with another promoter: SNP rs887829 ($r^2$=0.96) [9]. Interestingly, both SNPs were strongly linked with the functional TA$_n$ promoter polymorphism ($r^2$ = 0.88) and were significantly associated with TSB levels in Asian and European GWAS studies [8, 10]. Therefore, the protective effects of rs6742078 may be attributed to the strong LD between this site and the (TA)$_n$ repeat.

SLCO1B1 and SLCO1B3 genes are members of the OATP family, which is highly expressed in the basolateral membrane of hepatocytes. Among the other 6 SNPs in the other 4 bilirubin metabolism genes, only rs2306283 in SLCO1B1 and rs2117032 in SLCO1B3 showed a suggestive association with neonatal hyperbilirubinemia risk. Our finding was in accordance with recent case-control studies in Brazilian and Chinese neonates [35, 36], although two previous GWASs reported discordant results for the two loci of SLCOs on the TSB levels between Korean- and European-derived populations [8, 10]. In addition, the high MAF of rs2306283 and rs2117032 in the current cohort suggested that variants in SLCOs alone might not account for the substantial increased hyperbilirubinemia risk. In another words, there may be an additive effect of SLCO variants and other icterogenic conditions. Together, they were correlated with a significantly increased risk hyperbilirubinemia. This hypothesis was partly supported by Huang et al.’s observation in Taiwan neonates, who reported a significant additive effect of UGT1A1*6 variant and OATP1B1*1b on neonatal hyperbilirubinemia.

Heme oxygenase-1 (HO-1; OMIM*141250) is the initial and rate-limiting enzyme in the conversion of heme to bilirubin. It is believed that a short (GT)$_n$ repeat might be associated with a higher TSB levels and thus influence the hyperbilirubinemia risk in newborns [18]. This hypothesis is supported by two case-control studies [37, 38]. Both studies illustrated a significantly increased risk for the development of hyperbilirubinemia in neonates carrying short alleles compared to those carrying longer alleles. However, we did not find any relationship between the (GT)$_n$ repeat polymorphism and the hyperbilirubinemia risk in our study cohort, even when we tried different cut-off values to define the ‘short’ and ‘long’ allele classes of the (GT)$_n$ repeats. This was consistent with sequential studies by Zhou et al. [20, 32] in Chinese neonates, and Sato et al. on severely jaundiced Japanese neonates [31] The latter group also
reported a lack of an association between the short (GT)<sub>n</sub> repeat variant and an increased risk of neonatal hyperbilirubinemia. Furthermore, Kaplan et al in a population of Israeli neonates showed that (GT)<sub>n</sub> repeat length did not modulate bilirubin metabolism and TSB level of neonates at the 3 postnatal day [39]. We did not have enough information to explain these discrepant findings. However, these results may suggest that HO-1 is not the major gene involved in the pathogenesis of neonatal hyperbilirubinemia. Therefore, the effects of HMOX1 on the TSB levels could easily be affected by different genetic backgrounds [7] and other icterogenic conditions [40].

We acknowledge that this study has some limitations. The retrospective sampling employed here, which shaped the clinical distribution of the cohort and the sample size, may have limited our ability to definitively identify common variants with relatively small effect sizes of the expected type. For instance, we did not find strong evidence for the role of common variation in the SLCO family on neonatal hyperbilirubinemia risk as we initially evaluated the genetic effect based on the absolute occurrence of significant hyperbilirubinemia. Ideally, when we further divided the study neonates by the severity of their jaundice, the significant effects of the two risk predictors could have been observed. Indeed, this partly suggests that a quantitative approach could significantly increase the statistical power for the detection of genetic factors [41]. The validity of categorizing the study neonates into the 3 above mentioned groups could be questioned. The model fitting information shown in Table 4 showed that the ordered models were appropriate.

Another limitation was that the SNPs employed in the present study did not encompass all common variations at UGT1A1, HO-1, BLVRA, SLCO1B1, and SLCO1B3. However, the present study was economical and included almost all important and known functional common variants in the five bilirubin metabolism genes reported in the Asian population. This would provide a framework for the larger and more comprehensive evaluation of susceptibility genes.

Severe neonatal hyperbilirubinemia is a complex pediatric disorder. The usual studied clinical factors alone cannot provide the real cause. Genetic variance is responsible for this condition. Future study will further clarify the interactions among multiple bilirubin metabolism gene loci, other genes, and nongenetic factors to neonatal hyperbilirubinemia.

In conclusion, this detailed analysis revealed the impact of multiple genetic modifiers on neonatal hyperbilirubinemia, reflecting the complex nature of neonatal hyperbilirubinemia. The data obtained in this study has notably increased our knowledge on the molecular pathogenesis of neonatal hyperbilirubinemia and has provided genetic markers for clinical risk assessment. Furthermore, the established HRM for genotyping common mutations and polymorphisms could be used as a general and rapid method for future large-scale investigation.

**Supporting Information**

S1 Table. Primers used for genotyping the 11 common polymorphisms across the five bilirubin metabolism genes. (DOC)

S2 Table. Linkage disequilibrium (LD) analysis of the UGT1A1 gene. (DOC)

S3 Table. Detailed clinical information of control neonates described in manuscript. (XLS)
Author Contributions
Conceived and designed the experiments: QW LY LZ HY. Performed the experiments: HY ML XZ FL. Analyzed the data: HY LY ML LZ. Contributed reagents/materials/analysis tools: QW. Wrote the paper: HY.

References
1. Bhutani VK, Johnson LH, Jeffrey Maisels M, Newman TB, Phibbs C, Stark AR, et al. Kernicterus: epidemiological strategies for its prevention through systems-based approaches. J Perinatol 2004; 24:650–662. PMID:15254556
2. Kaplan M, Bromiker R, Hammerman C. Severe neonatal hyperbilirubinemia and kernicterus: are these still problems in the third millennium? Neonatology 2011; 100:354–362. doi:10.1159/000330055 PMID:21968213
3. Watchko JF, Daoood MJ, Biniwale M. Understanding neonatal hyperbilirubinaemia in the era of genomics. Semin Neonatol 2005; 10:113–122. doi:10.1016/j.siny.2005.02.002 PMID:15647632
4. Lin Z, Fontaine J, Watchko JF. Coexpression of gene polymorphisms involved in bilirubin production and metabolism. Pediatrics 2008; 122:e156–162. doi:10.1542/peds.2007-3249 PMID:18558634
5. Kaplan M, Muraca M, Hammerman C, Rubalentei FF, Vilei MT, Vreman HJ, et al. Imbalance between production and conjugation of bilirubin: a fundamental concept in the mechanism of neonatal jaundice. Pediatrics 2002; 110:e47. PMID:12359820
6. Lin R, Wang X, Wang Y, Zhang F, Wang Y, Fu W, et al. Association of polymorphisms in four bilirubin metabolism genes with serum bilirubin in three Asian populations. Hum Mutat 2008; 30:609–615. doi:10.1002/humu.20895 PMID:19243019
7. Johnson AD, Kavousi M, Smith AV, Chen MH, Dehghan A, Aspelund T, et al. Genome-wide association meta-analysis for total serum bilirubin levels. Hum Mol Genet 2009; 18:2700–2710. doi:10.1093/hmg/ddp202 PMID:19414484
8. Sanna S, Busonero F, Maschio A, McArdle PF, Usala G, Dei M, et al. Common variants in the SLCO1B3 locus are associated with bilirubin levels and unconjugated hyperbilirubinemia. Hum Mol Genet 2009; 18:2711–2718. doi:10.1093/hmg/ddp203 PMID:19419973
9. Kang TW, Kim HJ, Ju H, Kim JH, Jeon YJ, Lee HC, et al. Genome-wide association of serum bilirubin levels in Korean population. Hum Mol Genet 2010; 19:3672–3678. doi:10.1093/hmg/ddq281 PMID:20639394
10. Kringen MK, Piehler AP, Grimholt RM, Opdal MS, Haug KB, Urdal P. Serum bilirubin concentration in healthy adult North-Europeans is strictly controlled by the UGT1A1 TA-repeat variants. PLoS One 2014; 9:e90248. doi:10.1371/journal.pone.0090248 PMID:24587300
11. Editorial Board of Chinese Journal of Pediatrics; Subspecialty Group of Neonatology, The Society of Pediatrics, Chinese Medical Association. Experts consensus on principles for diagnosis and treatment of neonatal jaundice. Zhonghua Er Ke Za Zhi 2010; 48:685–686. [Article in Chinese] PMID:21092529
12. Pan M, Lin M, Yang L, Wu J, Zhan X, Zhao Y, et al. Glucose-6-phosphate dehydrogenase (G6PD) gene mutations detection by improved high-resolution DNA melting assay. Mol Biol Rep 2013; 40:3073–3082. doi:10.1007/s11033-012-2381-6 PMID:23275194
13. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 1992; 48:361–372. PMID:1637966
14. Solé X, Guinó E, Valls J, Iniesta R, Moreno V. SNPStats: a web tool for the analysis of association studies. Bioinformatics 2006; 22:1928–1929. PMID:16720584
15. SPSS Inc. SPSS advanced models. Chicago IL: SPSS Inc. 1999.
16. Yamada N, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, et al. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am J Hum Genet 2000; 66:187–195. PMID:10631150
17. Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, et al. Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum Genet 2002; 111:1–8. PMID:12196229
20. Zhou Y, Wang SN, Li H, Zha W, Wang X, Liu Y, et al. Association of UGT1A1 Variants and Hyperbilirubinemia in Breast-Fed Full-Term Chinese Infants. PLoS One 2014; 9:e104251. doi: 10.1371/journal.pone.0104251 PMID: 25102181

21. Newman TB, Xiong B, Gonzales VM, Escobar GJ. Prediction and prevention of extreme neonatal hyperbilirubinemia in a mature health maintenance organization. Arch Pediatr Adolesc Med 2000; 154:1140–1147. PMID: 11074857

22. Huang CS, Chang PF, Huang MJ, Chen ES, Hung KL, Tsou KI. Relationship between bilirubin UDP-glucuronosyl transferase 1A1 gene and neonatal hyperbilirubinemia. Pediatr Res 2002; 52:601–605. PMID: 12357057

23. Kang H, Lim JH, Kim JS, Kim ER, Kim SD, Lee HJ, et al. The Association of Neonatal Hyperbilirubinemia with UGT1A1 and CYP1A2 Gene Polymorphism in Korean Neonates. Korean J Pediatr 2005; 48:380–386.

24. Zhou YY, Lee LY, Ng SY, Hia CP, Low KT, Chong YS, et al. UGT1A1 haplotype mutation among Asians in Singapore. Neonatology 2009; 96:150–155. doi: 10.1159/000209851 PMID: 19325249

25. Chou HC, Chen MH, Yang HI, Su YN, Hsieh WS, Chen CY, et al. 211 G to a variation of UDP-glucuronosyl transferase 1A1 gene and neonatal breastfeeding jaundice. Pediatr Res 2011; 69:170–174. doi: 10.1203/PDR.0b013e31820263d2 PMID: 20975617

26. Ota Y, Maruo Y, Matsui K, Mimura Y, Sato H, Takeuchi Y. Inhibitory effect of 5α,3β-pregnane-3α,20β-diol on transcriptional activity and enzyme activity of human bilirubin UDP-glucuronosyltransferase. Pediatr Res 2011; 70:453–457. doi: 10.1038/pr.2011.678 PMID: 21796020

27. Fujiwara R, Nguyen N, Chen S, Tukey RH. Developmental hyperbilirubinemia and CNS toxicity in mice humanized with the UDP glucuronosyltransferase 1 (UGT1) locus. Proc Natl Acad Sci U S A. 2010; 107:5024–5029. doi: 10.1073/pnas.0913290107 PMID: 20914756

28. Fujiwara R, Chen S, Karin M, Tukey RH. Reduced expression of UGT1A1 in intestines of humanized UGT1 mice via inactivation of NF-κB leads to hyperbilirubinemia. Gastroenterology 2012; 142:109–118.e2. doi: 10.1053/j.gastro.2011.09.045 PMID: 21983082

29. Dai X, Wu C, He Y, Gui L, Zhou L, Guo H, et al. A genome-wide association study for serum bilirubin levels and gene-environment interaction in a Chinese population. Genet Epidemiol 2013; 37:293–300. doi: 10.1002/gepi.21711 PMID: 23371916

30. Long J, Zhang S, Fang X, Luo Y, Liu J. Association of neonatal hyperbilirubinemia with uridine diphosphate-glucuronosyltransferase 1A1 gene polymorphisms: Meta-analysis. Pediatrics International 2011; 53:530–540. doi: 10.1111/j.1442-200X.2011.03337.x PMID: 21342357

31. Sato H, Uchida T, Toyota K, Kanno M, Hashimoto T, Watanabe M, et al. Association of breast-fed neonatal hyperbilirubinemia with UGT1A1 polymorphisms: 211G>A (G71T) mutation becomes a risk factor under inadequate feeding. J Hum Genet 2013; 58:7–10. doi: 10.1038/jhg.2012.116 PMID: 23014115

32. Zhou Y, Wang SN, Li H, Zha W, Peng Q, Li S, et al. Quantitative trait analysis of polymorphisms in two bilirubin metabolism enzymes to physiologic bilirubin levels in Chinese newborns. J Pediatr 2014; 165:1154–1160. doi: 10.1016/j.jpeds.2014.08.041 PMID: 25262300

33. Skierka JM, Kotzer KE, Lagerstedt SA, O’Kane DJ, Baudhuin LM. UGT1A1 genetic analysis as a diagnostic aid for individuals with unconjugated hyperbilirubinemia. J Pediatr 2013; 162:1146–1152, 1152.e1–2. doi: 10.1016/j.jpeds.2012.11.042 PMID: 23290513

34. Beutler E, Gelbart T, Demina A. Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? Proc Natl Acad Sci U S A 1998; 95:8170–8174. PMID: 9653159

35. Alencastro de Azevedo L, Reverbel da Silveira T, Carvalho CG, Martins de Castro S, Giugliani R, Matte U. UGT1A1, SLCO1B1, and SLCO1B3 polymorphisms vs. neonatal hyperbilirubinemia: is there an association? Pediatr Res 2012; 72:169–173. PMID: 22580719

36. Liu J, Long J, Zhang S, Fang X, Luo Y. Polymorphic variants of SLCO1B1 in neonatal hyperbilirubinemia in China. Ital J Pediatr 2013; 39:49. doi: 10.1186/1824-7288-39-49 PMID: 24090270

37. Tiwari PK, Sethi A, Basu S, Ramam R, Kumar A. Heme oxygenase-1 gene variants and hyperbilirubinemia risk in North Indian newborns. Eur J Pediatr 2013; 172:1627–1632. doi: 10.1007/s00431-013-2091-7 PMID: 23877636

38. Katayama Y, Yokota T, Zhao H, Wong RJ, Stevenson DK, Taniguchi-Ikeda M, et al. Association of HMOX1 gene promoter polymorphisms with hyperbilirubinemia in the early neonatal period. Pediatr Int 2015; doi: 10.1111/ped.12591

39. Kaplan M, Renbaum P, Hammerman C, Vreman HJ, Wong RJ, Stevenson DK. Heme oxygenase-1 promoter polymorphisms and neonatal jaundice. Neonatology 2014; 106:323–329. doi: 10.1159/000365744 PMID: 25277974
40. Bozkaya OG, Kumral A, Yesilirmak DC, Ulgenalp A, Duman N, Ercal D, et al. Prolonged unconjugated hyperbilirubinaemia associated with the haem oxygenase-1 gene promoter polymorphism. Acta Paediatr 2010; 99:679–683. doi: 10.1111/j.1651-2227.2009.01678.x PMID: 20121710

41. Hanchard NA, Skierka J, Weaver A, Karon BS, Matern D, Cook W, et al. UGT1A1 sequence variants and bilirubin levels in early postnatal life: a quantitative approach. BMC Med Genet 2011; 12: 57. doi: 10.1186/1471-2350-12-57 PMID: 21513526