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ORIGINAL ARTICLE

Variants of organic anion transporter polypeptide 2 gene are not risk factors associated with severe neonatal hyperbilirubinemia

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Abstract

Objectives: This study aimed to determine the prevalence of four variants of organic anion transporter polypeptide 2 (*OATP2*) gene, and their association with severe hyperbilirubinemia. *Design:* Observational study. *Setting:* A tertiary university unit. *Patients:* Term infants of Chinese descent. *Methods:* 175 infants, consisting of 65 admitted for treatment of severe hyperbilirubinemia (with serum bilirubin levels > 250 mmol/L at age 1-2 days or > 300 μmol/L at age ≥ 3 days) and 110 randomly selected inborn infants without severe hyperbilirubinemia during their first month of life, were recruited. Their blood samples were subjected to sequencing analysis of exon 4 and exon 5 of OATP2 gene for detection of c.388A>G, c.521T>C, c.571T>C and c.597C>T variants. *Results:* The c.388A>G variant was the most common, and the c.521 T>C was least common, being present in 90.9% and 26.9% of the infants, respectively. Forward logistic regression analysis showed that the only significant risk factors associated with severe hyperbilirubinemia among these Chinese infants were: exclusive breast feeding (adjusted odds ratio (OR) = 12.5, 95% C.I.: 2.9, 53.4; p=0.001), infants with homozygous 211 variant of the *UDPG 1A1* gene (adjusted OR = 37.7, 95% C.I.: 4.4, 324.1; p=0.001), and G6PD enzyme level <8.5 IU/g Hb (adjusted OR = 7.3, 95% C.I.: 3.1, 17.5; p<0.00001). Gestational age, *G6PD* mutation status, actual G6PD enzyme level, and the 4 variants of the *OATP2* gene mutation were not significant risk factors. *Conclusion:* Variants of *OATP2* gene were not significant risk factors associated with severe hyperbilirubinemia in Malaysian Chinese infants.

Keywords: OATP2 variants, severe neonatal hyperbilirubinemia

INTRODUCTION

Organic anion transporter polypeptide 2 (OATP2), also known as OATP-C, OATP1B1, and LST -1, is responsible for the transportation of organic anions into hepatocytes. This mechanism may also be involved in the transportation of unconjugated bilirubin.[1] High prevalence of c.388A>G (73.4%) and c.521T>C (14.0%) variants of OATP 2 gene has been reported among Chinese in mainland China.[2] Besides these mutations, Jada *et al* and Nishizato *et al* have reported high frequency of two other silent mutations in OATP2 gene in Asian population.[3,4] Variations at c.571T>C and c.597C>T were detected in 26% and 50%, respectively, of a Chinese population,[3] and 50.0% and 42.9%, respectively, of a Japanese population.[4,5] A study from Taiwan showed that variation at c.388A>G was a risk factor associated with unconjugated hyperbilirubinemia in newborns.[3]

In Malaysia, neonatal hyperbilirubinemia is a common problem. The prevalence of OATP2 variants and its possible association with severe neonatal hyperbilirubinemia has not been studied in Malaysian Chinese populations previously. This study aimed to determine the prevalence of these variations and whether they were significantly associated with severe hyperbilirubinemia in the Malaysian infants of Chinese descent.

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TABLE 1: Primers used for sequencing analysis of organic anion transporter 2 (*OATP 2*) gene

Primer OATP2	
Exon4F	5'-TCTTTCTTGCTGGACACTTCC-3'
Exon4R	5'-GTGTTGTTAATGGGCGAACTG-3'
Exon5F	5'-GCAGCATAAGAATGGACTAATACAC-3'
Exon5R	5'-TACTAGATGCCAAGAATGCATGG-3'

METHODS

This was an observational study of 175 Malaysian normal term infants of Chinese descent. They included 65 infants who were admitted to the neonatal intensive care unit for treatment of severe hyperbilirubinemia (defined as serum bilirubin levels > 250 μmol/L at age 1-2 days or > 300 μmol/L at age 3 days and onward) and 110 randomly selected inborn infants without severe hyperbilirubinemia based on follow-up during the first month of life. Infants with conjugated hyperbilirubinemia > 15% of total bilirubin levels, age ≥ 28 days and premature infants < 36 weeks gestation were excluded. No patient had sepsis, gastrointestinal obstruction, polycythemia, or congenital malformation. No neonate developed kernicterus in this study. Parental consent was obtained and the Research and Ethnics Committee, Faculty of Medicine, University Kebangsaan Malaysia approved this project.

DNA extraction

Total genomic DNA extraction was isolated from ethylenediaminetetraacetic acid-anticoagulated blood samples by standard techniques using GeneAll DNA Extraction kit (General Biosystems, Seoul, Korea).

Sequencing analysis of OATP2 gene

All samples were subjected to sequencing analysis for exon 4 and exon 5 of OATP2 gene in order to identify the c.388A>G, c.521T>C, c.571T>C and c.597C>T variant alleles. The primers for sequencing analysis are listed as in Table 1. High Resolution Melting (HRM) analysis was used to genotype c.388A>G and c.521T>C. Primers for the HRM analysis were designed using Beacon Designer version 5.11. Table 2 lists the sequence of primers employed in performing HRM to genotype c.388A>G, and c.521T>C. Two separate 20 μl polymerase chain reactions (PCR) were carried out. PCR amplification and HRM analysis were performed on the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). Each of the PCR reaction consisted of 25 ng of DNA as template, 1X Takara Premix Buffer for Perfect Real Time, 5 μM of SYTO 9 dye and 150 nM of forward and reverse primers. All PCR reactions were performed in duplicates. The first PCR reaction (for c.388A>G) was run according to the following conditions: 40 cycles of 95°C for 10s, 50°C for 10s and 72°C for 15s, a melt from 74°C to 84°C rising at 0.2°C per second. The second PCR reaction (for c.521T>C) was run according to the following conditions: 40 cycles of 95°C for 10s, 56°C for 10s and 72°C

TABLE 2: Primers used for high resolution melting analysis to genotype c.388A>G, and c.521T>C variants of Organic Anion Transporter 2 (*OATP2*) Gene

SLCO1B1/OATP2 SNP	Primer sequence	Fragment size
A388G	F- 5'-ATTCAGTGATGTTCTTACAGTTAC-3'	133BP
	R- 5'-CTATCTCAGGTGATGCTCTATTG – 3'	
T521C	F- 5' –TTGTTTAAAGGAATCTGGGTCA-3'	77BP
	R- 5' – TACCTAAATACAAAGAAGAATG-3'	

for 15s, a melt from 74°C to 84°C rising at 0.2°C per second. As a quality control measure, samples showing amplifications after 30 cycles or more with Ct value of more than 30 (due to too little starting template amount or template degradation effects) and samples which reached the plateau phase after 35 cycles were excluded from further analysis. Melting curves data were then automatically normalized and displayed as normalized melting curve and difference plots. Genotyping for c.571T>C and c.597C>T were performed with Taqman minor groove binder (MGB) single neucleotide polymorphism (SNP) genotyping assay (Applied Biosystems, Foster City, CA).[6]

Serum bilirubin levels of jaundiced infants during the study was measured by the diazo method using the Cobas Integra system (Roche, Switzerland).[7] Quantitation of G6PD enzyme level in erythrocytes was determined by the ultraviolet method[8] using Randox glucose-6-phosphate dehydrogenase kits (Randox laboratories Ltd, United Kingdom) and a spectrophotometer (model 717, Hitachi, Japan). G6PD deficiency was diagnosed when an enzyme level was <8.5 IU/g Hb, based on our previous study.[9] Detection of G6PD variants and UGT1A1 variant at exon 1 (211G→A mutation) was by real-time polymerase chain reaction (PCR) with Taqman MGB SNP genotyping assay (Applied Biosystems, Foster City, CA), using ABI PRISM SDS 7000 (Applied Biosystems, Foster City, CA).[9,10]

Data analysis

The Statistical Package for Social Science (SPSS) version 11.5 (Chicago, IL, U.S.A.) was used for analysis of data. Variables between infants with and without severe hyperbilirubinemia were compared. The Student's t test (unpaired) was used for analysis of continuous variables with normal distribution. The Chi square test (or Fisher's exact test for expected values of less than 5) was used for analysis of categorical variables. Using severity of jaundice as the dependent variables and gestational age, types of feeds, 4 variants of OATP2 gene, G6PD mutation, actual G6PD enzyme levels, G6PD enzyme level < 8.5 IU/g Hb and 211 variant of UGT1A1 gene as independent variables, forward logistic regression analysis was carried out to determine the significant risk factors associated with severe hyperbilirubinemia in all infants. Variables were retained in the model if p values were <0.05. The goodness of fit of the model was checked using the Hosmer Lemeshow test.

RESULTS

The 175 infants included in the study consisted of 98 (56%) males, and 77 (46%) females. Their mean birth weight was 3048 g (SD = 437) and their mean gestational age was 38.6 weeks (SD = 1.2).

All genotyping results from both assays for OATP genes were in complete concordance with sequencing result. Table 3 shows that c.388A>G variant was the most common of the four variants of *OATP* gene, as it was present in 90.9% of the infants, and more than half of them were homozygote. Variant c.521 T>C was the least common, being present in only 26.9% of the infants, and none of them were homozygote.

Among the 110 infants without severe hyperbilirubinemia, 41.8% (n = 46) did not develop jaundice at all during the first month of life and 58.2% (n = 65) had only mild jaundice with a mean peaked total serum bilirubin of 238 μmol/L (SD = 39). The mean peaked serum bilirubin level of the 65 infants with

TABLE 3: Frequency distribution of infants of Malaysian Chinese Descent According to Four Different Variants of Organic Anion Transporter 2 (*OATP2*) Gene

Types	*OATP2* Variants			
	c.388 A>G n=175 (%)	c.521 T>C n=175 (%)	c.571 T>C n=175 (%)	c.597 C>T n=175 (%)
Homozygote	93 (53.1)	0 (0)	14 (8.0)	59 (33.7)
Heterozygote	66 (37.7)	47 (26.9)	61 (34.9)	83 (47.4)
Wild Type	16 (9.1)	128 (73.1)	100 (57.1)	33 (18.9)

TABLE 4: Relationship between hyperbilirubinemia status and demographic data, types of feeds, G6PD status, *UDPG 1A1* variants and *OATP2* variants of normal term infants of Chinese descent

Variables	Severe Hyperbilirubinemia n=65	No Severe Hyperbilirubinemia n=110	P values
Mean birthweight, g (SD)	3043 (377)	3051 (470)	0.9
Mean gestation, weeks (SD)	38.3 (1.4)	38.7 (1.1)	0.03*
Males (%)	39 (60)	59 (53.6)	0.4
Types of feeding (%)			
Exclusive breast feeding	14 (21.5)	3 (2.8)	<0.0001*
Mixed feeding	36 (55.4)	74 (67.9)	
Formula only	15 (23.1)	32 (29.4)	
G6PD mutation (%)			
Homozygous	2	1	
Heterozygous	15 (26.2)	5 (5.5)	<0.0001*
Wild type	48 (73.8)	104 (94.5)	
Mean G6PD enzyme levels, IU/gHb (SD)	9.6 (5.7)	11.8 (3.1)	0.006*
G6PD enzyme level <8.5, IU/gHb (%)	23 (35.4)	11 (10)	<0.0001*
UDPG 1A1 mutation (%)			
Homozygous	10 (15.4)	1 (0.9)	0.001*
Heterozygous	16 (24.6)	26 (23.6)	
Wild type	39 (60)	83 (75.5)	
OATP2 genes (%)			
Genotype c.338A>G			
Homozygous	38 (58.5)	55 (50.0)	0.2
Heterozygous	19 (29.2)	47 (42.7)	
Wild type	8 (12.3)	8 (7.3)	
Genotype c.521T>C			
Heterozygous	18 (27.7)	29 (26.4)	0.8
Wild type	47 (72.3)	81 (73.6)	
Genotype c. 571 T>C			
Homozygous	5	9	
Heterozygous	15 (30.8)	46 (50.0)	0.01*
Wild type	45 (69.2)	55 (50.0)	
Genotype c.597 C>T			
Homozygous	22 (33.8)	37 (33.6)	0.8
Heterozygous	32 (49.2)	51 (46.4)	
Wild type	11 (16.9)	22 (20.0)	
Total number of OATP risk alleles (%)			
1-2	18 (27.7)	20 (18.2)	0.1
3-5	47 (72.3)	90 (81.8)	
No. of OATP genes with homozygous mutations (%)			
0	19 (29.2)	42 (38.2)	0.3
1	27 (41.5)	33 (30.0)	
2	19 (29.2)	35 (31.8)	

Note: G6PD= glucose-6-phosphate dehydrogenase, UDPG 1A1= uridine diphosphoglucuronyl transferase 1A1, OATP = organics anion transporter protein, SD= standard deviation, * denotes statistical significance.

severe hyperbilirubinemia was 347 μmol/L (SD = 45) at a mean age of 6.3 days (SD = 3.3). Univariate analysis showed that there was no significant difference in the mean birthweight and gender distribution between infants with and without severe hyperbilirubinemia (Table 4). Although all the infants were of term gestation, those with severe hyperbilirubinemia had significantly lower gestation. When compared with infants without severe hyperbilirubinemia, a significantly higher proportion of infants with severe hyperbilirubinemia were exclusively breast fed, had *G6PD* mutation, had G6PD enzyme level <8.5 IU/g Hb, and had homozygous mutation of the *UDPG1A1* gene ($p < 0.01$). Their mean G6PD enzyme levels were also significantly lower than infants without severe hyperbilirubinemia. There was no significant difference in the proportion of infants with mutation of the c.388A>G variant, c.521 T>C variant, and c.971C>T variant of the OATP2 gene. However, a significantly lower proportion of infants with severe hyperbilirubinemia had mutation of the c.571 T>C variant than infants without severe hyperbilirubinemia ($p = 0.01$). There was no significant difference in the number of risk alleles and the number of homozygous mutation between the two groups of infants (Table 4).

Forward logistic regression analysis showed that, after controlling for various potential confounders, the only significant risk factors associated with severe hyperbilirubinemia among these Chinese infants were: exclusive breast feeding (adjusted odds ratio (OR) = 12.5, 95% C.I.: 2.9, 53.4; $p = 0.001$), infants with homozygous 211 variant of the *UDPG 1A1* gene (adjusted OR = 37.7, 95% C.I.: 4.4, 324.1; $p = 0.001$), and G6PD enzyme level<8.5 IU/g Hb (adjusted OR = 7.3, 95% C.I.: 3.1, 17.5; $p < 0.00001$). Gestational age, *G6PD* mutation status, actual G6PD enzyme level, and the four variants of the *OATP2* gene mutation were not significant risk factors.

DISCUSSION

The results of the present study showed that all four variants of the *OATP2* gene were common among Chinese infants in Malaysia. These findings are consistent with those of other investigators[2,3] who reported that c.388A>G variant of OATP2 was the most common in their population. The present study showed that c.388A>G variant of *OATPS* gene was highly polymorphic in Malaysian Chinese population as 92.7% of infants without severe hyperbilirubinemia and 74.2% of infants with severe hyperbilirubinemia were affected. Furthermore, this study also showed that the allelic frequency of c.521T>C variant (13.6% in not severe hyperbilirubinemic infants and 14.1% in severely hyperbilirubinemic infants) was similar to those reported in the Chinese (13%) in Taiwan and the Japanese population (16%).[3-5] No homozygosity for c.521T>C was detected in the present study. The allele frequency of c.571T>C (29.7%) and c.597C>T (55.0%) were comparable with those of Chinese and Japanese reported by other investigators.[3-5,11] The c.571T>C and c.597C>T variants of *OATP2* gene were generally regarded as silent mutations with no clinical importance. However, Kimchi-Sarfaty C *et al* showed that silent mutations were able to change the rate of protein folding and thus affect the affinity of protein towards substrate binding.[12] Future studies have been planned to investigate the impact of the various haplotypes, including the silent mutations, of *OATP2* gene on individual personalized drug therapeutics and pathogenesis of hereditary disease in the Malaysian population.

Similar to the findings of a case-control study reported by Huang *et al*,[13] we found that exclusive breast milk feeding was a significant risk factor. Contrary to their study, however, which reported c.388A>G variant of *OATP2* gene as a significant risk factor associated with hyperbilirubinemia, our study showed that none of the four variants of the *OATP2* gene were significant risk factors, after controlling for various potential confounders. Our findings concur with those of Wang *et al*[14] which reported that human OATP2 did not mediate bilirubin transport in OATP2 transfected cells in HeLa and HEK293 cell lines. However, our study suggests that the variant c.571T>C of *OATP2* gene may have a protective effect against the development of severe hyperbilirubinemia. Furthermore, unlike the study of Huang *et al*[13] which reported 211 variant of the *UGT1A1* gene to be a significant risk factor, our study showed that only the homozygous variant, not heterozygote variant of this gene was a significant risk factor. In their study population, the number of infants with G6PD deficiency was small (n=4). As a result, Huang *et al* did not identify G6PD deficiency as a significant risk factor unlike the finding in the present study.

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