Research paper

High resolution melting analysis of the NR1I3 genetic variants: Is there an association with neonatal hyperbilirubinemia?

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Abstract

Constitutive androstane receptor (CAR) encoded by the nuclear receptor subfamily 1, group I, member 3 (NR1I3) gene regulates the elimination of bilirubin through activating the components of the bilirubin clearance pathway. Hence, NR1I3 genetic variants may affect bilirubin metabolism and result in neonatal hyperbilirubinemia. Thus far, research which investigates the association between NR1I3 variants and neonatal hyperbilirubinemia has not been undertaken in any population. The present study aimed to evaluate the influence of MPJ6_1I3008 (rs10157822), IVS8+116T and 540A-G on neonatal hyperbilirubinemia development in the Malay population. Buccal swabs were collected from 232 hyperbilirubinemia and 277 control term newborns with gestational age ≥ 37 weeks and birth weight ≥ 2500 g. The NR1I3 variants were genotyped by using high resolution melting (HRM) assays and verified by DNA sequencing. Gender, mode of delivery and birth weight did not differ between hyperbilirubinemia and control groups. The genotypic and allelic frequencies were not significantly different between the groups. However, stratification by gender revealed a significant inverse association between homozygous variant genotype of MPJ6_1I3008 and risk of neonatal hyperbilirubinemia in the females (OR, 0.44; 95% CI, 0.20–0.95; p = 0.034). This study demonstrates that the homozygous variant genotype of MPJ6_1I3008 was associated with a significant reduced risk of neonatal hyperbilirubinemia in the females.

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1. Introduction

Neonatal hyperbilirubinemia or jaundice is a benign and common clinical manifestation of the newborns (American Academy of Pediatrics Subcommitte on Hyperbilirubinemia, 2004; Bhutani et al., 2010). The prevalence is 60% in term and 80% in preterm newborns (Rennie et al., 2010; The Lancet, 2010). Hyperbilirubinemia occurs due to an elevated production of bilirubin from senescent erythrocytes or reduced elimination of bilirubin from the body (Kaplan et al., 2002). Although neonatal hyperbilirubinemia is a natural phenomenon, an elevated serum bilirubin level has the potential to produce permanent brain damage (kermitcerus) or even death if it is not recognized and treated properly (Johnson et al., 2009).

Uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) is the key enzyme which conjugates bilirubin with glucuronic acid for the subsequent bilirubin elimination (Bosma et al., 1994). The promoter region of the UGT1A1 gene contains glucuronosyltransferase phenobarbital-responsive enhancer module (gtPBREM) that is regulated by constitutive androstane receptor (CAR) (Sugatani et al., 2001). In response to phenobarbital induction, CAR binds to the gtPBREM resulting in an elevated UGT1A1 transcription thereby enhancing the metabolism of bilirubin (Sugatani et al., 2001). Besides, other transcription factors which also regulate the transcription of UGT1A1 by binding to the gtPBREM include the pregnane X receptor (Sugatani et al., 2005), aryl hydrocarbon receptor (Yueh et al., 2003), nuclear erythroid-related factor 2 (Yueh and Tukey, 2007), peroxisome proliferator-activated receptor α (Senekeo-Effenberger et al., 2007) and glucocorticoid receptor (Sugatani et al., 2005).

CAR is an orphan nuclear receptor encoded by the nuclear receptor subfamily 1, group I, member 3 (NR1I3) gene (Baes et al., 2005).
2. Materials and methods

2.1. Study subjects

This was a comparative cross-sectional study involving subjects admitted to and/or born in Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia from April 2013 to October 2014. Ethical approval of the study was obtained from Human Research Ethics Committee of Universiti Sains Malaysia (No. USMKK/PPP/JEPEM[263.3.12]). Informed consent was obtained from the parents before recruiting a total of 509 subjects which comprised 232 hyperbilirubinemia and 277 non-hyperbilirubinemia newborns. Subjects from both groups were Malay term newborns with gestational age ≥ 37 weeks and birth weight ≥ 2500 g.

Newborns who developed total serum bilirubin (TSB) levels ≥ 250 μmol/L within the first week after birth were recruited into the hyperbilirubinemia group. Control subjects were newborns without significant hyperbilirubinemia (TSB levels < 250 μmol/L). Newborns who exhibited the following criteria were excluded from the study: prolonged hyperbilirubinemia, hemolysis, polycythemia, birth trauma, diabetic mother, ABO or Rh incompatibility, glucose-6-phosphate dehydrogenase (G6PD) deficiency, sepsis, asphyxia, congenital pneumonia, infection, gross congenital malformations and other medical conditions that could lead to neonatal hyperbilirubinemia.

2.2. Genomic DNA extraction and genotyping

Buccal swab, a non-invasive and cost-effective sampling tool was used to collect genomic DNA from the newborns. Briefly, two sterile buccal swabs were twirled gently on the inside of both cheeks for about 30 s. Genomic DNA was isolated using Exgene™ Blood SV mini kit (GeneAll, Korea) according to the manufacturer’s protocol. The extracted genomic DNA was stored at −20 °C prior to genotyping.

The NR1I3 nucleotide sequence was obtained from the National Center for Biotechnology Information (NCBI) (accession number: NG_029113.1). To genotype MPJ6_1I3008 (rs10157822), IVS8+116T→G (rs4073054) and 540A→G (rs2307424) single nucleotide polymorphisms (SNPs), high resolution melting (HRM) analysis was employed by using the PikoReal 96 real-time polymerase chain reaction (PCR) system (Thermo Fisher Scientific Inc., United States). The technique combined real-time PCR and post-PCR HRM in a single experiment. Primer pairs (Table 1) were designed with the aid of Primer3 web version 4.0.0 (Rozen and Skaltsky, 2000) and analyzed using PCR Primer Stats (Stothard, 2000) and NCBI BLAST (Altschul et al., 1990). Small amplicons (99–114 base pairs) were critical to increase the sensitivity of the HRM assays and to avoid multiple melting domains that would complicate the interpretation of the results.

The HRM assay required only a single preparation. Briefly, the HRM assay for each SNP was carried out in 10 μL of final volume in a 96-well Piko PCR white plate (Thermo Fisher Scientific Inc., United States): 5 μL of 2× Precision Melt Supermix (Bio-Rad Laboratories, Inc., CA) was added to 0.2 μL of each forward and reverse primer (10 μM; Sigma-Aldrich, Malaysia), 1 μL of DNA template (1 ng/μL) and 3.6 μL of distilled water.

The real-time PCR condition consisted of an initial denaturation step at 95 °C for 2 min that was followed by 40 cycles of denaturation (95 °C for 10 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). The annealing temperature (T_a) was optimized by gradually increasing or decreasing the T_a from 54 to 60 °C until an optimal HRM melt profile was obtained. Although the same T_a was used for all three primer pairs, HRM assay for each SNP was conducted separately to produce simple melt profiles that facilitated easy interpretation. The subsequent HRM program comprised heteroduplex formation (denaturation at 95 °C for 30 s and renaturation at 60 °C for 1 min) and high resolution melt with continuous fluorescence readings from 60 to 95 °C in 0.2 °C increments.

HRM data was analyzed by the PikoReal Software version 2.1 (Thermo Fisher Scientific Inc., United States). Genotypes of the samples were assigned by comparing the melting patterns with that of known standards. Samples constituting the same nucleotide sequence would demonstrate a similar melting pattern in the graphs. Hence, three different melting patterns were produced and that implied the three genotypes of a SNP.

2.3. DNA sequencing

To confirm the validity and reliability of the HRM genotyping results as well as to assign a genotype for each HRM pattern, representative samples demonstrating different melting patterns, random samples and those which generated unusual or ambiguous HRM results were subjected to DNA sequencing. A commercial kit (GeneAll® Expin™ Combo GP, Korea) was used to purify these samples prior to sequencing. The sequencing electropherograms were viewed by using Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and compared with the
NR1I3 sequence from NCBI to derive the genotype for each sequenced sample.

2.4. Statistical analysis

Statistical analysis was conducted by using IBM® SPSS statistics version 20 (SPSS, Chicago, IL, USA) and Epi Info 7 (Dean et al., 2011). To determine the significance of difference between the hyperbilirubinemia and control groups for the baseline demographic data and the frequencies of genotype and allele, Pearson's chi-square test was employed for categorical variables while independent t test was used to analyze numerical variables. The association between variant genotypes and risk of developing neonatal hyperbilirubinemia was explored by using binary logistic regression analysis with wild-type genotype served as the reference (odd ratio (OR) of 1.00). p value <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of study subjects

A total of 509 newborns were enrolled in this study, comprising 232 hyperbilirubinemia subjects with TSB $\geq$ 250 μmol/L and 277 control subjects with TSB < 250 μmol/L. The baseline demographic and clinical data were compared between the two subject groups. None of the newborn in either group had demonstrated any clinical condition that is critical for the development of neonatal hyperbilirubinemia. We did not find any significant difference in the gender, mode of delivery and birth weight between hyperbilirubinemia and control subjects. On average, the peak TSB level was observed on day four of life of the hyperbilirubinemia newborns. In these patients, their peak TSB levels were in the range of 250–324 μmol/L in 144 (62.1%) newborns and 81 (34.9%) newborns were between 325 and 399 μmol/L. Seven (3.0%) out of 232 hyperbilirubinemia newborns developed peak TSB ≥400 μmol/L which necessitated exchange transfusion.

3.2. NR1I3 variant analysis

A 100% concordance rate was achieved for the genotyping results produced by HRM and DNA sequencing analysis (data not shown). DNA sequencing electropherograms (Fig. 1) verified the nucleotide changes that were demonstrated by the characteristic HRM melting patterns. As shown in Figs. 2 and 3, distinctive HRM melting patterns were produced from different genotypes. DNA samples with identical nucleotide sequence were clustered into the same groups and exhibited similar melting patterns. Contrary, difference in the nucleotide was distinguished by melting temperature shift and changes in the melting curve shape. MPJ6_113008 (G$\rightarrow$A) and 540A$\rightarrow$G which involved adenine (A) and guanine (G) base variation generated similar melting patterns (Fig. 3). However, the melting temperature of the samples with comparable genotypes (for an example, GA of MPJ6_113008 versus AG of 540A$\rightarrow$G) was not identical due to different base compositions and lengths of the amplicons.

The frequencies of genotypes and alleles of MPJ6_113008, IVS8+116T$\rightarrow$G and 540A$\rightarrow$G were compared between the hyperbilirubinemia and control groups. The distributions of all variant genotypes and alleles between the groups were not significantly different ($p > 0.05$). The risk of developing neonatal hyperbilirubinemia for each NR1I3 variant genotype was estimated by logistic regression analysis (data not shown). The heterozygous and homozygous variant genotypes of all variants were positively associated with the neonatal hyperbilirubinemia risk (OR > 1.00), although the associations were

![Fig. 1. Segments of the DNA sequence electropherograms to characterize and confirm the genotype for each HRM melting pattern. Two peaks were observed in the electropherograms of heterozygotes. Genotypes are labeled above the electropherograms. Arrows indicate nucleotide changes.](image-url)
The development of neonatal jaundice is multifactorial which involves genetic and environmental components (American Academy of Pediatrics Subcommittee on Hyperbilirubinemia, 2004; Bhutani et al., 2004). To date, review of the literature revealed no studies which determined the association between variants in the NR1I3 gene and neonatal hyperbilirubinemia. In Malay newborns, we did not find any significant difference in genotypic distribution of MPJ6_1I3008, IVS8+116T>G and 540A>G between hyperbilirubinemia and control newborns. However, we found a significant inverse association between female newborns with homozygous variant genotype of MPJ6_1I3008 and risk of neonatal hyperbilirubinemia when gender-based stratification analysis was performed. The results suggest the protective role of homozygous variant genotype against neonatal hyperbilirubinemia in the female Malays.

To the best of our knowledge, comprehensive scanning of the NR1I3 variants was only conducted among the Japanese individuals administering drugs (Ikeda et al., 2003) and healthy Singapore Asians (Chew et al., 2013). The Japanese study identified a total of 29 SNPs including three SNPs that were reported previously (Ikeda et al., 2003). Among all the SNPs, 16 (including MPJ6_1I3008) were located in the 5′-flanking region, three in exons and ten in introns. Another study conducted on healthy Singapore Asians revealed a total of 38 SNPs and 13 out of which were novel ones (13 SNPs in 5′-flanking region, one in exon and 24 in introns) (Chew et al., 2013). The variant allele frequency of MPJ6_1I3008 in our population (43.3% and 47.3% in patients and controls respectively) was lower than the Japanese (66.4%) (Ikeda et al., 2003). This 5′-flanking SNP may have an effect on the promoter regulatory activity but the exact mechanism remains to be clarified. Variation in the distribution between the Malays and Japanese highlights the possible influence of ethnicity and background of subjects on genetic variation.

For IVS8+116T>G, we did not find any significant risk association for the heterozygous genotype in both genders, and the homozygous variant genotype in males. Although the homozygous variant genotype was not present in the female hyperbilirubinemia newborns, a reduced number (less than five) of the genotype was found in males and female controls. Stratification analysis of the 540A>G also revealed that in both genders, the association between neonatal hyperbilirubinemia risk and variant genotypes was not statistically significant.

4. Discussion

The development of neonatal jaundice is multifactorial which involves genetic and environmental components (American Academy of Pediatrics Subcommittee on Hyperbilirubinemia, 2004; Bhutani et al., 2004). To date, review of the literature revealed no studies which determined the association between variants in the NR1I3 gene and neonatal hyperbilirubinemia. In Malay newborns, we did not find any significant difference in genotypic distribution of MPJ6_1I3008, IVS8+116T>G and 540A>G between hyperbilirubinemia and control newborns. However, we found a significant inverse association between female newborns with homozygous variant genotype of MPJ6_1I3008 and risk of neonatal hyperbilirubinemia when gender-based stratification analysis was performed. The results suggest the protective role of homozygous variant genotype against neonatal hyperbilirubinemia in the female Malays.

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A study from Singapore showed that the homozygous variant genotype of IVS8+116T>G was not detected in 54 healthy Malay subjects and the variant allele frequency was 8.3% (Chew et al., 2013). However, both the heterozygous and homozygous variant genotypes were present in our subjects with a higher frequency (16.4%). The exonic SNP 540A>G (Pro180Pro) was mapped to the ligand binding domain of NR1I3 (di Masi et al., 2009). In-silico analysis showed that the silent
Fig. 3. HRM melting patterns generated from MPJ6_1I3008 (i and ii) and 540A–G (iii and iv) were almost similar. Both SNPs involved changes in A and G bases. There was a slight difference in the melting pattern of AA genotype between the SNPs.

Table 2
Stratification of neonatal hyperbilirubinemia risk association of NR1I3 variants by gender.

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
<th>Odd ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MPJ6_1I3008</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males (n)</td>
<td>133</td>
<td>136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (GG)</td>
<td>43 (32.3)</td>
<td>44 (32.4)</td>
<td>1.00 (reference)</td>
<td>–</td>
</tr>
<tr>
<td>Heterozygous (GA)</td>
<td>57 (42.9)</td>
<td>60 (44.1)</td>
<td>0.97 (0.56–1.69)</td>
<td>0.920</td>
</tr>
<tr>
<td>Homozygous variant (AA)</td>
<td>33 (24.8)</td>
<td>32 (23.5)</td>
<td>1.06 (0.55–2.01)</td>
<td>0.870</td>
</tr>
<tr>
<td>Females (n)</td>
<td>99</td>
<td>141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (GG)</td>
<td>35 (35.4)</td>
<td>37 (26.2)</td>
<td>1.00 (reference)</td>
<td>–</td>
</tr>
<tr>
<td>Heterozygous (GA)</td>
<td>50 (50.5)</td>
<td>70 (49.6)</td>
<td>0.76 (0.42–1.36)</td>
<td>0.348</td>
</tr>
<tr>
<td>Homozygous variant (AA)</td>
<td>14 (14.1)</td>
<td>34 (24.1)</td>
<td>0.44 (0.20–0.95)</td>
<td>0.034*</td>
</tr>
<tr>
<td><strong>IVS8+116T&gt;G</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males (n)</td>
<td>133</td>
<td>136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (TT)</td>
<td>93 (69.9)</td>
<td>91 (66.9)</td>
<td>1.00 (reference)</td>
<td>–</td>
</tr>
<tr>
<td>Heterozygous (TG)</td>
<td>36 (27.1)</td>
<td>41 (30.1)</td>
<td>0.86 (0.50–1.46)</td>
<td>0.576</td>
</tr>
<tr>
<td>Homozygous variant (GG)</td>
<td>4 (3.0)</td>
<td>4 (2.9)</td>
<td>0.98 (0.24–4.03)</td>
<td>0.976</td>
</tr>
<tr>
<td>Females (n)</td>
<td>99</td>
<td>141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (TT)</td>
<td>80 (80.8)</td>
<td>104 (73.8)</td>
<td>1.00 (reference)</td>
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</tr>
<tr>
<td>Heterozygous (TG)</td>
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<td>32 (22.7)</td>
<td>0.77 (0.41–1.46)</td>
<td>0.426</td>
</tr>
<tr>
<td>Homozygous variant (GG)</td>
<td>0 (0.0)</td>
<td>5 (3.5)</td>
<td>NA</td>
<td>0.052</td>
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<tr>
<td><strong>540A&gt;G</strong></td>
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<td></td>
</tr>
<tr>
<td>Males (n)</td>
<td>133</td>
<td>136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (AA)</td>
<td>35 (26.3)</td>
<td>43 (31.6)</td>
<td>1.00 (reference)</td>
<td>–</td>
</tr>
<tr>
<td>Heterozygous (AG)</td>
<td>63 (47.4)</td>
<td>58 (42.6)</td>
<td>1.33 (0.75–2.36)</td>
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<tr>
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<td>35 (25.7)</td>
<td>1.23 (0.64–2.35)</td>
<td>0.533</td>
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<tr>
<td>Females (n)</td>
<td>99</td>
<td>141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (AA)</td>
<td>24 (24.2)</td>
<td>37 (26.2)</td>
<td>1.00 (reference)</td>
<td>–</td>
</tr>
<tr>
<td>Heterozygous (AG)</td>
<td>58 (58.6)</td>
<td>71 (50.4)</td>
<td>1.26 (0.68–2.34)</td>
<td>0.465</td>
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<tr>
<td>Homozygous variant (GG)</td>
<td>17 (17.2)</td>
<td>33 (23.4)</td>
<td>0.79 (0.36–1.73)</td>
<td>0.562</td>
</tr>
</tbody>
</table>

Figures in parenthesis are percentage or 95% CI as applicable.

* Statistically significant.
coding SNP may regulate NR1I3 splicing by modulating the splicing factor (SRp55) binding (Chew et al., 2013). The variant allele frequency of the SNP in our study (47.8%) was comparable to those of the healthy Singapore Malays (45.3%) (Chew et al., 2013). Although the Malay populations from Singapore and Malaysia may share similar genetic composition, discrepancy in the frequencies may still exist due to different characteristics of the subjects, geographical factors and sample size.

A higher incidence of neonatal hyperbilirubinemia in the Asians compared with the Caucasians suggests that genetic factors could be the underlying cause for the disease occurrence (Huang et al., 2004; Morigaka et al., 2010). Genetic factors that predispose to neonatal hyperbilirubinemia including the UGT1A1, GPD1 and SLC01B1 variants have been extensively explored in various ethnicities (Watchko and Lin, 2010; Yusoff et al., 2010; Liu et al., 2013; D’Silva et al., 2014; Tiwari et al., 2014). Generally, these variants contribute to neonatal hyperbilirubinemia by disrupting the balance between bilirubin production and elimination (Kaplan et al., 2002). However, no studies have targeted the NR1I3 gene yet.

Since the protein product of NR1I3, CAR, regulates the bilirubin excretion by inducing important components (SLCO1B1, GSTA1, GSTA2, UGT1A1 and MRP2) involved in bilirubin clearance, and the ability of CAR to stimulate an enhanced bilirubin elimination in response to hyperbilirubinemia (Huang et al., 2003), therefore, it is possible that NR1I3 genetic variants which encode receptors with decreased amount or functions may affect the occurrence and severity of neonatal hyperbilirubinemia. The resulting CAR may demonstrate variable inductive or stimulatory activities on the related components. Additionally, the regulation of UGT1A1 transcription by CAR which binds to the gprBREM (Sugatani et al., 2001) also suggests that any abnormality in CAR due to the NR1I3 variants may modulate the binding efficiency and lead to a decreased production of UGT1A1 enzyme. Thus, the variants may indirectly reduce the metabolism of bilirubin and lead to neonatal hyperbilirubinemia. However, our findings demonstrated that female neonates with homozygous variant genotype of MPJ6_113008 have a lower risk of hyperbilirubinemia.

Extensive literature search shows that no studies have provided theoretical or functional explanation regarding the genetic mechanism of the protective role conferred by homozygous variant MPJ6_113008 against neonatal hyperbilirubinemia or other diseases. However, some other mutations demonstrate protective effects in which individuals with homozygous mutations are protected whereas heterozygous individuals may have delayed disease progression. For instance, hemoglobinopathies (hemoglobin C and hemoglobin E, sickle-cell with homozygous mutations are protected whereas heterozygous individuals demonstrate protective effects in which individuals have a lower risk of hyperbilirubinemia.

In conclusion, our results suggested a female-specific significant inverse association of the homozygous variant genotype of MPJ6_113008 with the risk of neonatal hyperbilirubinemia. Besides, MPJ6_113008, IVS5+116T–G and 540A–G were not significantly associated with the neonatal hyperbilirubinemia risk when compared between the groups in Malay population.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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