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Uridine Diphosphate Glucuronosyltransferase 1A1 (*UGT1A1*) Gene Polymorphisms in Indian Malaysian Newborns with Significant Neonatal Hyperbilirubinemia

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Abstract
Background*: The variations of the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) gene has been reported as risk factors associated with neonatal hyperbilirubinemia. Among UGT1A1 polymorphisms, the 211G>A, promoter A(TA)$_n$TAA and c.-3279T>G mutations are common in Asian population. However, studies on frequency and co-expression patterns of these variants in Indian Malaysian newborns with significant hyperbilirubinemiaare limited.*
Objective*: This study aims at determining the frequency pattern of 211G>A, promoter A(TA)nTAA and c.-3279T>G mutations in Indian Malaysian newborns with significant neonatal hyperbilirubinemia (SigNH).*
Methods*: A total of 1121 hyperbilirubinemic neonates wererecruited (Malay-Malaysian 74.9%, 839/1121, Chinese Malaysian 16.9%, 190/1121, Indian Malaysian 3.2%, 36/1121)from Selayang Hospital over an 18-month period. The inclusion criteria were all full-term infants admitted for treatment of hyperbilirubinemia. Dry blood spots (DBS) were collected from each of the infants. PCR-restriction fragment length polymorphism method was applied to detect six UGT1A1 gene variants of 211G>A, promoter A(TA)$_n$TAA, c.-3279T>G, 686C>A, 1091C>T, and 1456T>G mutations.*
Results*: Out of total 36 Indian Malaysian neonates studied (19 SigNH and 17 non-SigNH), the result of the present study showed that 63.9% (23/36) of neonates carried a UGT1A1 gene mutation.From the six variants ofUGT1A1 gene identified, three variant mutations were detected.The commonest allele was UGT1A1 promoter c.-3279T>G mutation (13 of 36) (SigNH=30.8%, non-SigNH=69.2%), followed by mutations of A(TA)$_7$TAA promoter (7 of 36) (SigNH=28.6%, non-SigNH=71.4%) and then the axon 1 mutation 211G>A (3 of 36) (SigNH=100.0%, non-SigNH=0.0%) which was undetected in previous reports. However, mutations of nucleotide 686C>A, 1091C>T, and 1456T>G were not detected in this study population.*
Conclusion*: Mutation of UGT1A1 gene at nucleotide 211G>Awas not only found in this Indian Malaysian cohort but was also significantly associated with SigNH indicating the potential of this mutation as a marker associated with higher risk for significant hyperbilirubinemia. Co-expression of c.-3279T>G and (TA)$_7$TAA promoter mutation was also found in this study cohort.*
Key words*: UGT1A1, significant neonatal hyperbilirubinemia*

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I. Introduction and background

Hyperbilirubinemia is the most common condition among neonates which requires evaluation and treatment. (1,2) Clinically, significant neonatal hyperbilirubinemia (SigNH)defined as total serum bilirubin (TSB) level ≥17 mg/dL (≥291 μmol/L) in term infants, leading to acute bilirubin encephalopathy that frequently evolved into a chronic stage known as kernicterus (3). Uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*) is the key enzyme for bilirubin conjugation, and mutations on this gene has been shown to be highly associated with unconjugated hyperbilirubinemia syndromes (4). The peak serum levels of unconjugated bilirubin in full-term Asian (Japanese, Korean or Chinese) and American Indian neonatesdoubles of those seen in Caucasian and black populations (5).A national survey conducted in Malaysia showed that 75% of newborns had jaundice in the first week of life, and 25-30% of these patients developed severe neonatal jaundice (TSB >342 umol/L)(6).Thus, incidence of kernicterus is shown to be higher among Asian infants(7, 8).

Several studies of *UGT1A1* mutations in Southeast Asian population have been reported (9,10), including Malaysia (11-15) in which the 211 G to A variation of *UGT1A1* gene was found to be the most common independent risk factor of neonatal hyperbilirubinemia(16) especially in Malay- Malaysian (12) and Chinese Malaysian (15). The effect of this mutation was indicated in another study in which the enzyme activity

of individuals with the c.211G>A mutation of heterozygous and homozygous status decreased to 60% and 32.2% respectively compared to that of normal(17).

The pattern of TA repeat mutation was found in hyperbilirubinemic (3.5%) and the control group (0.01%) in Malay-Malaysian (15)and was more commonly detected compared to the 211 mutation (9).A homozygous polymorphism in promotor region of the *UGT1A1* gene in which dinucleotide (TA) is inserted in the TATA box-like sequence, leading to an $A(TA)_7TAA$ nucleotide sequence was shown in unconjugated hyperbilirubinemic patients particularly with Gilbert syndrome (18). In addition, *UGT1A1* enzyme activity was lowest in hepatic tissue from the homozygote for the variant $A(TA)_7TAA/A(TA)_7TAA$ (7/7) promoter and intermediate in the $A(TA)_6TAA/A(TA)_7TAA$ (6/7) heterozygote, compared with the normal $A(TA)_6TAA/A(TA)_6TAA$ (6/6) homozygote (19).

Yusoff*et al* reported that the frequency of homozygosity for c.-3279G was much higher in patients than those in controls of Malaysian neonates(13). The *UGT1A1* c.-3279T>G gene mutation decreases the transcriptional activity of the *UGT1A1* promoter by 60% (20) and the reduction of U*GT1A1* activity was shown to cause Crigler-Najjar syndrome (21).

On the other hand, studies involving Indian neonates from India showed that *UGT1A1* gene variant 211G>A, was identified as an independent molecular risk factor for neonatal hyperbilirubinemia (22) whereas TATA box mutation and variant c.-3279T>G were notably found to be significantly associated with severe neonatal hyperbilirubinemia and identified as independent risk factors for neonatal hyperbilirubinemia of Indian newborns (23,24).

Although there were studies done in *UGT1A1* gene mutations in Malaysia, the frequency patternsof especially variants of 211G>A, promoter $A(TA)_nTAA$, c.-3279T>G have not been highlighted in Indian Malaysian jaundicednewborns. Similarly, co-expression on *UGT1A1* variant mutations has not been reported in Indian Malaysian neonates.Hence, this paper highlights key patterns of UGT1A1 gene mutations and co-expression in Indian Malaysian hyperbilirubinemic newborns.

II. Methods

SigNH and non-SigNH subjects. This was a retrospective analysis of all the newborns of Indian ethnic group recruited in a large sample study of 1121 multi-racial Malaysian jaundiced term newborns admitted for phototherapy to a large Malaysia hospital (25). In the present study, we investigated the prevalence of *UGT1A1* in these Indian Malaysian neonates and to determine whether *UGT1A1* variants were risk factors associated with clinically significant neonatal hyperbilirubinemia (TSB≥291 µmol/L).All parents of neonates gave consent for their infants to participate in this study, which was approved by Medical Research and Ethics Committee (MREC) of Selayang Hospitals and the UniversitiTunku Abdul Rahman Research Committee.

Determination of *UGT1A1* gene. For the determination of *UGT1A1* gene status, total genomic DNA was isolated from dry blood spot (DBS) sample, ten punches of 1.2 mm spots from the DBS were subjected to DNA extraction using DNA extraction protocol (Bioline Inc, U.S.A). The PCR-restriction fragment length polymorphism (PCR-RFLP) method was applied to detect the known variant sites in the *UGT1A1* gene 211G>A, promoter $A(TA)_nTAA$, c.-3279T>G, 686C>A, 1091C>T, and 1456T>G. The PCR mixture (25 ul) consisted of 200 ng of DNA, 1 ul of each primer (20 uM each), 12.5 ul of Mytaq Mix (Bioline Inc, U.S.A) and 8.5 ul of water (ddH_2O). The PCR amplification was performed in a DNA thermal cycler (applied Biosystems, Veriti, U.S.A) for 35 cycles of initial denaturation for 1 min at 95 °C, annealing for 15 sec at 55-59 °C, primer extension for 10 sec at 72 °C. The PCR product was digested with the appropriate restriction enzyme and analyzed on 3% agarose gel (NHK Bioscience Solutions SdnBhd, Malaysia). For confirmation of results performed with PCR-RFLP method, sequences of *UGT1A1* gene in DNA samples were determined. All the results were matched with those determined by the sequencing method (Applied Biosystems3730XLDNA Analyzer andApplied Biosystems Sequence Scanner Software for sequence result analysis, U.S.A).

Statistical analysis

Statistical analysis was conducted by using IBM SPSS Statistic 22.0 for Windows software program 2013 (SPSS Inc., Chicago, IL, USA). Data were expressed as percentage for categorical variables.Fisher's exact test was used for comparisons of frequencies of male and female and *UGT1A1* variants between SigNH and non-SigNH babies. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for hyperbilirubinemia. A 95% CI for OR≥1.0 or P-value of <0.05were considered statistically significant.

III. Results

Thirty-six (3.2%, 36/1121)neonates were from Indian ethnic group among the whole sample recruited in the study. Demographic, genotypic distribution and co-expression of *UGT1A1* gene in SigNH and non-SigNH groups are shown in Table 1. There were 9 boys and 10 girls in SigNH group and 6 boys and 11 girls in non-

SigNH group respectively with no significant difference in their frequencies in between two groups [OR: 0.61 (95%CI: 0.16-2.32; p=0.46)].

The result of the present study showed that 63.9% (23/36) of the Indian ethnic group of hyperbilirubinemic neonates carried at least one*UGT1A1* mutation.The overall frequency of occurrence of the variant c.-3279T>G of *UGT1A1* gene mutation was observed to be significantly higher in non-SigNH compared with SigNH (9 vs 4 cases) with OR: 4.03 (95%CI: 0.06-1.02; p=0.05), with higher heterozygous cases than in homozygous in the former group. Similarly, a higher proportion of newborns in the non-SigNH group had variation of $(TA)_7$ promoter compared with that of the SigNH group (5 vs 2 cases) with OR: 3.76 (95%CI: 0.05-1.71; p=0.15). Of these 7 samples, 3 are heterozygous and 4 are homozygous cases but neither $(TA)_5$ nor $(TA)_8$ variant were found.

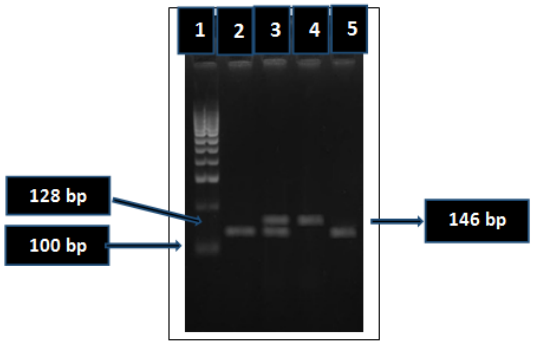
However, there was no significant difference in the frequency of $(TA)_7$ polymorphisms in between two groups. Variant c.211G>A was identified to be significantly associated with SigNH with OR: 0.49 (95%CI: 0.34-0.69; p=0.04). There were 3 co-expression cases of A$(TA)_7$TAA and c.-3279T>G mutations identified in the non-SigNH group [OR: 3.66 (95%CI: 0.29-0.63; p = 0.06)]. We did not detect any mutations of nucleotide 686C>A, 1091C>T, and 1456T>G in our study population.

Table 1. Demographic, Genotypic distribution and Coexpression of *UGT1A1* mutations in the study

Category	*n = 36*		OR (95% CI)	P-value
	Non-SigNH (TSB<291 umol/L) (*n* = 17)	SigNH (TSB≥291 umol/L) (*n* = 19)		
Gender				
Male	6 (40.0%)	9 (60.0%)	0.61 (0.16-2.32)	0.46
Female	11 (52.4%)	10 (47.6%)		
c.-3279T>G				
T/T	8 (34.8%)	15 (65.2%)	0.50 (0.26-1.01)	
T/G	6 (66.7%)	3 (33.3%)	4.03 (0.06-1.02)	0.05*
G/G	3 (75.0%)	1 (25.0%)	2.12 (0.89-5.05)	
A(TA)nTAA				
TA)6/(TA)6	12 (41.4%)	17 (58.6%)	0.28 (0.05-1.71)	
(TA)6/(TA)7	3 (100.0%)	0 (0.0%)	3.76 (0.05-1.71)	0.15
(TA)7/(TA)7	2 (50.0%)	2 (50.0%)	1.01 (0.61-6.88)	
211G>A				
G/G	17 (51.5%)	16 (48.5%)	2.13 (0.07-67.80)	
G/A	0 (0.0%)	1 (100.0%)	0.49 (0.34-0.69)	0.04*
A/A	0 (0.0%)	2 (100.0%)	0.50 (0.01-49.57)	
Coexpression				
(TA)7/(TA)7 and c.-3279T/G	1 (100.0%)	0 (0.0%)	3.66 (0.29-0.63)	0.06
(TA)7/(TA)7 and c.-3279G/G	2 (100.0%)	0 (0.0%)		

* p<0.05

Note: All zero count was continuity corrected with 0.5.



Lane 1: Ladder 100 bp
Lane 2 and 5: GG homozygote
Lane 3: GA heterozygote
Lane 4: AA homozygote

Fig 1. PCR and restriction pattern of (211G>A) *UGT1A1* gene. Restriction fragment pattern of PCR product after digesting with *Ava II* and running in 3% agarose gel

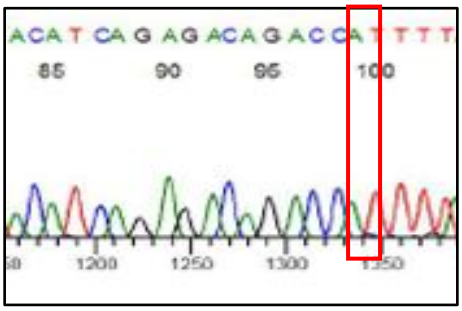
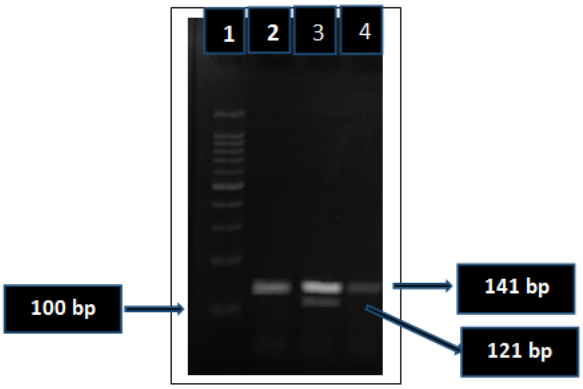


Fig 2. Sequence chromatogram of (211G>A) *UGT1A1* gene shows G71R mutation



Lane 1: Ladder 100 bp
Lane 2 and 4: TT homozygote
Lane 3: TG heterozygote

Fig 3. PCR and restriction pattern of (3279T>G) *UGT1A1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 4 are restriction fragment pattern of PCR products after digesting with *Dra I* and running in 3% agarose gel

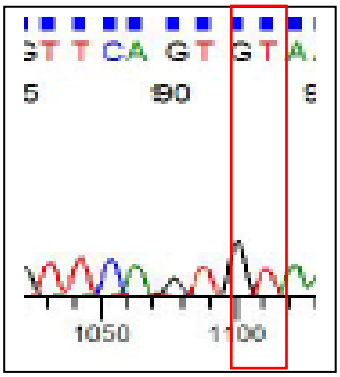


Fig 4. Sequence chromatogram of *UGT1A1* gene variant c.-3279T>G showing GG homozygous mutation

IV. Discussion

The frequency of SigNH is 52.8% (19/36) in the study cohort and the frequency of c.-3279T>G variant was the highest among the three variants identified and, interestingly, the frequency was significantly higher in non-SigNH than in SigNH population. The allele frequency reported on this mutation was 0.55 in Indians (22), 0.50 in Malays (13), 0.49 in Americans (26), 0.34 in Chinese (27), 1.00 in Indonesians (28), and 0.26 in Japanese (29).Yusoff*et al* found that c.-3279T>G variant as a significant risk factor for severe neonatal hyperbilirubinemia in Malay neonates(13).However, the exact pathogenesis of c.-3279T>G variant mutation in hyperbilirubinemia remains to be further elucidated (22).

In this study, promoter A$(TA)_n$TAA mutation was found to have higher frequency in non-SigNH group (n=5, 71.4%) but no significant difference was found between SigNH and non-SigNH. This TA repeat mutation was also found to have higher frequency in other studies (18,22-23).A$(TA)_n$TAA mutation reduces

transcriptional activity of *UGT1A1* gene to 60-80% of normal (30) and it is the most common mutation associated with Gilbert syndrome in the Indian (31) and Western (32) populations.

Variant 211 G>A (Gly71Arg) mutation in the axon 1 of *UGT1A1* gene was detected in SigNH from 3 out of 36 (8.3%) individuals sampled in this study. Although this mutation was identified in hyperbilirubinemic Indian Malaysian neonates (4/62, 6.5%), there was no significant difference in case and control groups (11).The decreased *UGT1A1* enzyme activities are thought to cause delayed elimination of bilirubin and ultimately occurrence of hyperbilirubinemia (33). This current study was the first report of the 211 G>A variant mutation in Indian Malaysian newborns with significant hyperbilirubinemia. Moreover, interestingly. the homogenous mutation of nt 211 G>A mutation with Indian neonate was presented with severe degree of neonatal hyperbilirubinemia (TSB >342 mmol/L) (25).

There were two cases of homozygous (TA)7/(TA)7 and homozygous c.-3279G/Gand one case of homozygous (TA)7/(TA)7 and heterozygous c.-3279T/G co-expression wereidentified in non-SigNH group. One study showed that the patients with c.-3279T>G polymorphisms are also important for the decrease of transcription of *UGT1A1* in addition to $A(TA)_7TAA$, and that Gilbert syndrome is likely to be caused by the combined effects of these polymorphisms (34). Munro *et al* reported that homozygosity for c.-3279T>G and $A(TA)_7TAA$ may be associated with neonatal jaundice and/or Gilbert syndrome (35). According to Ferraris *et al* (36), homozygosity for both c.-3279T>G and $A(TA)_7TAA$ was associated with the highest relative risk estimate (OR = 19.23; $p < 0.001$) and they believed that there is a synergistic effect of c.-3279T>G and $A(TA)_7TAA$ and calculated that the association of these two mutations could lower the transcriptional activity of *UGT1A1* to the Gilbert syndrome causing level. In addition, Li *et al* (37) stated that the combined genotypes containing A(TA)7TAA and -3279T>G decreases *UGT1A1* transcription.

As the demographic distribution of the sample collection (25) showed that Indian Malaysian has the smallest number of cases during the recruitment period and the small sample size of the individuals of Indian origin may reflect a limitation of this study. Nevertheless, the presence of *UGT1A1* gene variants in the Malaysian individuals of Indian origin described in this study is crucial in addressing the overall impact of its association with significant neonatal hyperbilirubinemia.

V. Conclusion

Mutation of *UGT1A1* gene at nucleotide 211G>Awas not only found in this study cohort Indian Malaysian neonate but was also significantly associated with SigNHsuggesting that the Indian Malaysian neonates who carry variant 211G>A in the *UGT1A1* gene are potentially at higher risk for significant hyperbilirubinemia. Furthermore, the occurrence of c.-3279T>G allele was the highest among the variants detected and significantly higher in non-SigNH group. Although the $A(TA)_7TAA$ promoter mutation did not show a significant difference between the SigNH and non-SigNH, it was found to be co-expressed with 3279T>G.

Author's contribution

Shwe Sin was principal author who conceptualized the manuscript, wrote background, methods, results and discussion of the manuscript, performed data analysis, interpretation of the results, and revised the final drafts of the manuscript.

Ong HK and Boo NY assisted in conceptualization of the manuscript and data analysis.

Jabbar MA assisted inanalyzing and revisionof the manuscript data.

Chee SK, Maslina M, Miclelle L, Kaur A performed sample collection and demographic data collection.

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Competing interests: none

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