

ORIGINAL ARTICLE

Prevalence of uridine glucuronosyl transferase 1A1 (UGT1A1) mutations in Malay neonates with severe jaundice

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Abstract

A number of genetic risk factors have been implicated in the development of neonatal severe hyperbilirubinaemia. This includes mutations in the uridine glucuronosyl transferase 1A1 (UGT1A1) gene which is responsible for unconjugated hyperbilirubinemia in Gilbert's Syndrome. We studied the prevalence of UGT1A1 gene mutations in a group of Malay neonates to determine whether they are risk factors to severe neonatal jaundice. One hundred and twenty-five Malay neonates with severe hyperbilirubinemia were studied. Ninety-eight infants without severe hyperbilirubinaemia were randomly selected from healthy Malay term infants (controls). DNA from EDTA cord blood samples were examined for UGT1A1 mutations nt211G>A and nt247T>C using established Taqman SNP genotyping assays and the UGT1A1*28 variant was detected by the Agilent 2100 bioanalyzer. All samples were also screened for common Malay G6PD variants using established techniques. The frequency of UGT1A1 211G>A mutation is significantly higher in the severely hyperbilirubinemic group (13%) than the control group (4%; $p=0.015$) and all the positive cases were heterozygous for the mutation. There was no significant difference in the frequency of UGT1A1*28 mutation between the severely hyperbilirubinemic (3.5%) and the control group (0.01%; $p=0.09$). None of the neonates in both groups carried the nt247 T>C mutation. The prevalence of G6PD mutation was significantly higher in the severely jaundiced group than control (9% vs 4%; $p=0.04$). In conclusion, nt 211 G>A alleles constitute at least 12% of UGT1A1 mutations underlying unconjugated hyperbilirubinemia and appears to be a significant independent risk factor associated with severe neonatal hyperbilirubinemia in the Malay newborns.

Key words: UGT1A1 mutation, severe hyperbilirubinaemia, neonatal jaundice

INTRODUCTION

Neonatal jaundice is a common and serious condition as 25-30% of these neonates develop severe neonatal jaundice (serum bilirubin > 300 $\mu\text{mol/L}$) which may lead to kernicterus. There are many recognized causes of severe neonatal jaundice, however, a significant 44% of cases show no obvious risk factor.¹ The incidence of severe hyperbilirubinaemia and kernicterus is higher among newborn Asian infants compared to Caucasians suggesting an underlying genetic factor being responsible for the development of neonatal jaundice.^{2,3}

In addition to G6PD deficiency, mutations in the uridine glucuronosyl transferase 1A1

(UGT1A1) gene have been shown to be associated with prolonged unconjugated hyperbilirubinemia in newborn infants. Aono *et al*⁴ in 1995 were the first to demonstrate that mutation for UGT1A1 was responsible for patients with Gilbert's syndrome, a mild form of UGT1A1 deficiency characterized by intermittent unconjugated hyperbilirubinemia that generally manifests after puberty. Subsequent to this, TA repeat promoter polymorphism in the UGT1A1 gene, (UGT1A1*28) was reported to be associated with prolonged neonatal hyperbilirubinemia in Scottish newborns and associated with increased in the incidence of neonatal hyperbilirubinemia in G6PD- deficient Sephardic Jews.⁵ However, a mutation in the coding region at nt 211 G>A

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that results in G71R amino acid change has been shown to be associated with severe unconjugated hyperbilirubinemia in the Taiwanese, the Japanese and the Koreans.^{6,7,8} A study on a group of Malaysian Chinese neonates showed that nt 211 G>A is a significant risk factor associated with severe hyperbilirubinemia.⁹ The aim of our study was to determine whether known mutations of the UGT1A1 gene that are associated with Gilbert's syndrome exist in the Malay population and whether they are risk factors associated with severe hyperbilirubinemia. We present here our study on the prevalence of the UGT1A1 nt 211 G>A mutation, UGT1A1*28 variant and nt 247 T>C mutation in Malay neonates with severe hyperbilirubinemia and non-hyperbilirubinemic neonates. We have included mutation nt 247 T>C because it was recently shown to cause Gilbert's syndrome in a Thai family¹⁰ and based on the notion that Malays and Thais may share the same ethnic origin.

MATERIALS AND METHODS

A cross-sectional observational study was carried out over eighteen months (July 2006–December 2007) on neonates in the postnatal ward and babies admitted to neonatal intensive care unit, Hospital Universiti Kebangsaan Malaysia. The inclusion criteria for hyperbilirubinemia newborns were term neonates (>38 weeks gestation and 42 completed weeks) of Malay ethnic origin who were admitted for severe unconjugated neonatal hyperbilirubinaemia (Total serum bilirubin (TSB) > 250umol/L for neonates aged 1 to 2 days of life or TSB > 300umol/L for neonates aged more than 3 days). Cord blood samples were collected from the haematology laboratory which routinely receives screening samples for G6PD from the labour rooms. Neonates with conjugated hyperbilirubinemia >15% TSB levels, age > 28 days of life, those born prematurely (<36 weeks), babies born to mixed parentage and neonates with conditions which were likely to exacerbate hyperbilirubinaemia such as ABO incompatibility, cephalhaematoma and sepsis were also excluded from the study. Healthy term infants of Malay ethnicity born in the hospital were recruited consecutively as controls. All control cases were followed-up via phone call within the first month after discharge from the ward. Informed consent was obtained from parents of the infants prior to participation in the study. The study protocol was approved by the Institutional Research and Ethics Committee.

Bilirubin testing

Serum bilirubin was measured using Cobas Intergra 800 analyzer using the colorimetric assay for serum total bilirubin and Diazo method for direct bilirubin quantification.

DNA extraction

DNA was extracted from cord blood leucocytes using the Gene All DNA kit (General Biosystems, Seoul, Korea) according to the manufacturer's instruction and was kept at -20°C until required. Blood DNA samples were analysed for the nucleotide 247 polymorphism and the UGT1A1*28 variant of the UGT1A1 promoter region by Taqman single nucleotide polymorphism (SNP) genotyping assay. Primers and probes were designed using Beacon Designer Software Version 5.0 (Biosearch Technologies, Inc.) (Table 1) based on UGT1A1 exon 1 (nt247 T>C mutation) and the TATA box gene sequences obtained from www.ncbi.nlm.nih.gov.

Genotyping of 247 (T>C) mutation

For the 247 (T>C) mutation, Real Time Quantitative PCR were optimized for each reaction using the following concentrations; a final reaction of 25 µL which consisted of Quantitect Multiplex PCR NoROX Master Mix (Qiagen) 12.5 µL, wild type and mutant probes, 1.5 µL each, forward and reverse primers 1.5 µL and distilled water 5.5 µL. PCR amplification and detection was performed using Corbett Life Science Rotor-gene 6000 (Corbett Research, Australia). The amplification programme was carried out for 40 cycles (activation of Taq polymerase for 10 minutes at 95°C before denature, denature for 10 seconds at 95°C, annealing for 15 seconds at 52°C and extend for 20 seconds at 72°C. Allelic discrimination was performed on the post PCR product. The fluorescence data of the post PCR product were analysed directly with the allelic discrimination software preinstalled in the Rotor-Gene 6000.

*Genotyping of UGT1A1*28 variant*

For the UGT1A1*28 variant, the PCR were optimised for each reaction using the following concentration; a final reaction of 20.0 µl which consisted of Qiagen High Fidelity Master Mix (Qiagen) 10.0 µl, forward and reverse primers 1.0 µL and distilled water 6.0 µL. PCR amplification was performed using the Gene AMP PCR System 9700 (Applied Biosystems). The amplification programme was carried out for 35 cycles (Initial activation of Taq polymerase

TABLE 1: Primers, probes and platform used for 247 (T>C) mutation and TA variant

Mutation	Forward primer	Reverse primer	Probe for wild type allele	Probe for mutant allele	Platform
247 (T>C)	5' GTC CTA GCA CCT GAC G 3'	5' AAA CAT TAT GCC CGA GAC 3'	5' CAL Fluor Gold 540- TAC ATC AGA GAC GGA GCA TTT TACA- BHQ-1 3'	5' FAM- TAC ATC AGA GAC AGA GCA TTT TACA-BHQ-1 3'	Corbett Life Science Rotor-gene 6000 (Corbett Research, Australia)
A(TA) ₇ TAA variant	5' CAC CTT CTT TAT CTC TGA AAG TG 3'	5' TCT CAG ATA CCA AGA AAT CAT CC 3'	-	-	Agilent 2100 bioanalyzer

for 2 minutes at 94°C before denature, denature for 20 seconds at 94°C, annealing for 10 seconds at 53°C and extend for 20 seconds at 72°C and a final extension for 2 minutes at 72°C. 1uL PCR products was subjected to the Agilent Bioanalyzer using the 1000 LabChip kit.

Genotyping of 211 G>A mutation and G6PD variants

SNP analysis for the 211 G>A mutation and three G6PD variants (C1024T, G871A, G487A and C563T) for our Malay neonates were performed as described in a previous study.¹¹ Detection of G6PD variants was by real-time polymerase chain reaction (PCR) with Taqman minor groove binder (MGB) SNP genotyping assay (Applied Biosystems, Foster City, CA). The fluorescence data of the post-PCR products were analyzed directly using allelic discrimination software of the ABI Prism 7000 instrument.

DNA Sequence Analysis

For confirmation of the real-time PCR findings, all samples were subjected to sequencing using primers sequenced for G6PD exon 2–13 as described previously.¹¹

Statistical analysis

SPSS for Windows (version 12.0) statistical package software was used for the statistical analysis of the study. The chi squared test was used for analysis of categorical variables. P

value of <0.05 is considered as statistically significant.

RESULTS

A total of 250 neonates were enrolled in this study. One hundred and twenty-five neonates of Malay ethnic parentage were admitted with severe unconjugated hyperbilirubinemia and fulfilled the inclusion criteria for the study. Four infants were excluded from the study due to inadequate blood samples and/or poor DNA quality. A total of 98 normal term Malay neonates born in the hospital were recruited consecutively for the non-hyperbilirubinemic group. Twenty-three neonates were non-hyperbilirubinaemic but developed clinically moderate jaundice at the median age of 4 days and were excluded from the analysis. Serum bilirubin was measured in the non-hyperbilirubinemia infants with clinically moderate jaundice. All non-hyperbilirubinaemic infants were discharged home within four days after birth and none developed severe hyperbilirubinemia during the first week of life. None of them was readmitted during the subsequent 3 weeks of life for severe hyperbilirubinemia or other illnesses.

The mean peak total indirect serum bilirubin of the severely jaundiced group was 348.5 umol/l ± 47.7 SD (n=125) and for the non-hyperbilirubinaemic infants with clinically moderate jaundice was 211.3 umol/l ± 53.25 SD (n=23). The mean age for the onset of jaundice

was 3.7 days \pm 1.9 SD and the mean age in days for bilirubin to peak was 5.6 days \pm 3.7 SD. There was an equal distribution of gender between the severely jaundice neonates and the control cases. There was no significant difference in the gender distribution and the mean birth weight between the hyperbilirubinemic group and the non-hyperbilirubinemic newborns.

All the cases carrying the mutation nt 211 were heterozygous for the mutation with a prevalence

of 10% (22/223). The overall prevalence of UGT1A1*28 mutation was found to be 2% (5/223) and that of the G6PD mutations was found to be 10% (22/223). No homozygous nt 211 G>A was found. The UGT1A1 gene mutation nt 211 G>A (13% vs 4.2%; $p=0.01$) (Figure 1) and G6PD mutations 10% (13/125) vs 4% (4/98) $p=0.04$ were found to be significantly higher in the severely jaundiced group than the control group respectively but not for

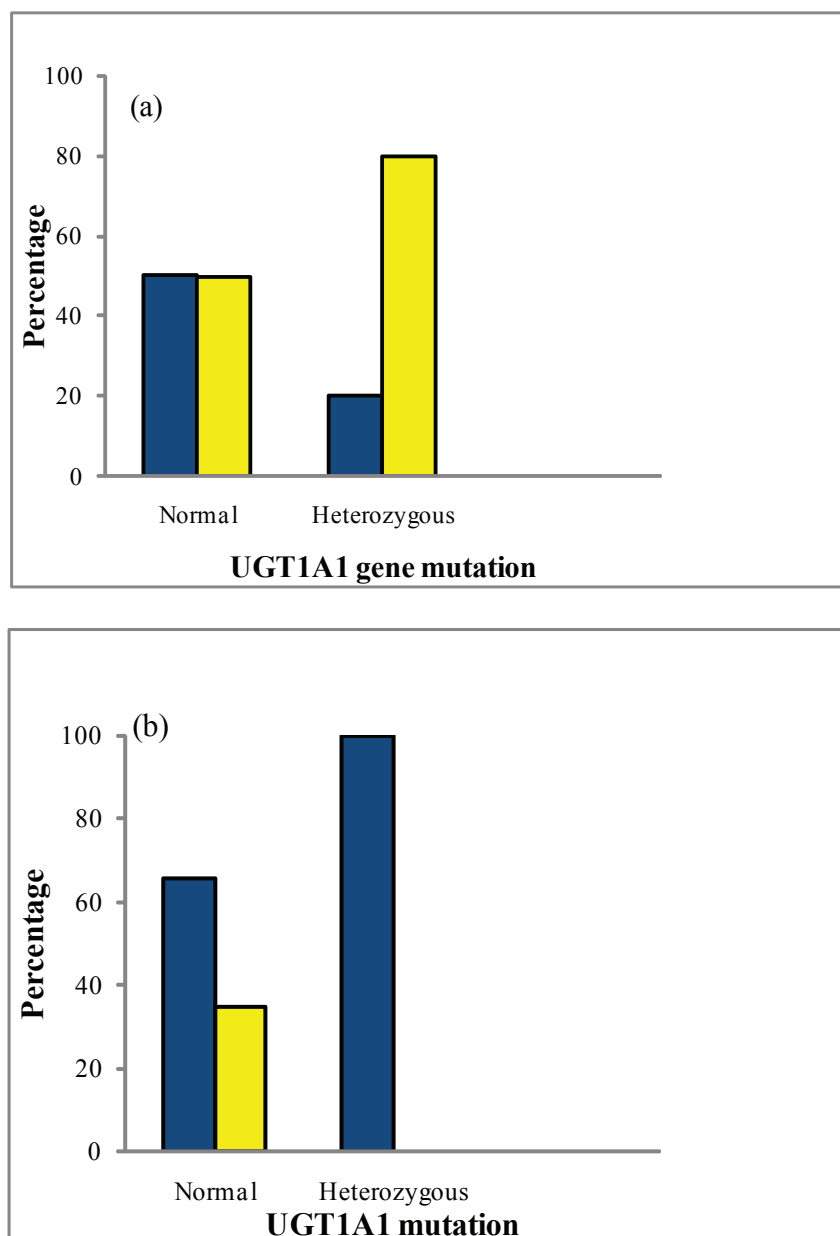


FIG. 1: Comparison of prevalence of nucleotide 211 G>A mutation of uridine diphosphate glucuronosyl transferase (UGT1A1) gene between non-hyperbilirubinaemic (■) and hyperbilirubinaemic (■) newborns in relation to (a) absence ($p<0.01$) and (b) presence of glucose-6-phosphate dehydrogenase deficiency.

UGT1A1*28 mutation. The allelic frequency of UGT1A1*28 was found to be low in both the severely jaundiced and the control group (3.5% vs 0.01%; $p=0.09$). None of the 223 babies from both groups showed presence of the nt 247 T>C mutation. Fourteen out of 22 (64%) cases positive for G6PD mutation were of the Viangchan variant (871 G>A). Twenty two out of 223 samples analysed showed presence of G6PD mutations (2 female heterozygotes, 11 female homozygotes and 9 male hemizygotes). Coinheritance of UGT1A1 mutation (nt 211 G>A) and G6PD mutation was only seen in one case of severe neonatal jaundice.

DISCUSSION

The result of the present study showed that at least 12% of the Malay neonates carried a UGT1A1 mutation, the gene responsible for Gilbert's Syndrome. Out of the 3 known mutations screened the commonest mutation found was nt 211 G>A (10%) with the TA promoter region repeat (UGT1A1*28) occurring in only 2%. Our finding is comparable to that of Surini et al where it was found that the prevalence of UGT1A1*28 in a group of Malays was 1.5%.¹² However, in contrast to Surini's study where they found the prevalence of nt 211 G>A was low, we found nt 211 G>A to be the commonest allele showing a frequency of 10%. Mutation nt 211 G>A is an allele common in Asian populations of Chinese and Japanese descent. One previous study by our group confirmed the association of mutation nt 211 G>A in the Chinese when we found 26% of Malaysian Chinese neonates carried the mutation. The same study on 74 Chinese neonates found that homozygous nt 211 G>A is a significant risk factor associated with severe hyperbilirubinemia.⁹ Previous studies in G6PD deficiency¹³ and thalassaemias^{14,15,16} have shown that although the Malays and the Chinese differ in their genetic profile there were presence of some Chinese-specific G6PD mutations in the Malay population reflecting ethnic admixtures most likely due to the intermarriages. Retno et al studied the frequency of nt 211 G>A in Javanese-Indonesians and Malay-Malaysians and found it to be low.¹⁷ The finding of a frequency of 10% mutation nt 211 G>A in the Malays may be explained by the effect of ethnic admixtures. However, what is important in our finding is that there is a significant difference in the prevalence of nt 211 G>A between the hyperbilirubinemic group (13%) and the control group (4.2% $p=0.01$) suggesting its role as a

risk factor to severe neonatal unconjugated hyperbilirubinemia in Malay neonates. In contrast to two previous studies where homozygous nt 211 G>A was shown to be the associated risk factor to severe jaundice, in the present study we found that all the cases positive for the mutation were heterozygous nt 211 G>A.^{3,9} Boo et al showed that the prevalence of nt 211 G>A in the hyperbilirubinemic infants was 39.2% and the control group was 25.6%, $p=0.04$. The findings of heterozygous nt 211 G>A as a significant risk factor for severe neonatal jaundice in the Malay population could be explained by co-existence of other undetermined genetic risk factors that remained to be explored. Huang et al showed that G6PD, UGT1A1 and OATP2 gene mutations were significant risk factors to severe neonatal hyperbilirubinemia.

The low occurrence of UGT1A1*28 at 2% (5/223) in the Malays is expected as this has been shown to be a common mutation causing Gilbert's syndrome in Caucasians. It was observed that all the four positive cases in the severely jaundiced group were heterozygous for the UGT1A1*28. Homozygous UGT1A1*28 does not appear to cause hyperbilirubinemia in the single normal infant found to be genotypically homozygous. These findings suggest that other factors may play a role in the development of severe jaundice in those heterozygous for UGT1A1 TA promoter mutation.

The present study confirmed our previous findings that G6PD mutation is the risk factor to severe neonatal jaundice (hyperbilirubinemic vs non-hyperbilirubinemic : 10% vs 4%, $p=0.04$) and that the commonest G6PD variant in the Malays is G6PD Viangchan (14/22). All the 16 cases of severe neonatal jaundice with nt 211 G>A were normal for G6PD screening test and this includes 6% of males and 7% of females. Out of 13 cases with severe neonatal jaundice who carried a G6PD mutation 11/13 were normal for G6PD screening test and this include 2 male and 9 female neonates. The four (3 males and 1 female) severely jaundiced babies heterozygous for the UGT1A1*28 had normal G6PD screening test results and did not carry any of the common G6PD mutation suggesting interplay of other unknown risk factors. In the hyperbilirubinemic group there was only one infant who carried both G6PD mutation (Viangchan variant) and the UGT1A1 nt 211 G>A mutation. These findings showed that G6PD mutation and heterozygous UGT1A1 nt 211 G>A mutation are independent risk factors to severe neonatal jaundice. Huang

et al observed that the development of severe hyperbilirubinemia in G6PD-deficient male is due to coinheritance with homozygous variant in UGT1A1 gene. These findings suggest in addition to G6PD status, G6PD and UGT1A1 mutations should be included in screening for genetic risk for severe hyperbilirubinemia in our newborn.

Previous studies in Thalassaemias and G6PD deficiency have shown that Malays and ethnic Chinese differ in their genetic profile and that the Malays do share some common mutations with other Southeast Asian population such as the Thais, Cambodians and the Vietnamese. Based on this notion we screened for UGT1A1 247 T>C variant a mutation that was reported in two Thai siblings with a history of prolonged neonatal jaundice and recurrent jaundice after infancy. We found that none of our Malay subjects either in the case or the control group was positive for this variant.

In the present study we report the use of Agilent 2100 bioanalyzer for the detection of the UGT1A1*28. The software that comes with the analyzer was able to display results in the form of an electropherogram and a corresponding gel image. The different alleles can be distinguished by looking at the electropherogram for the presence of a single peak and its size (wild type and homozygous alleles) or the presence of a bi-peak (homozygous allele) or it can also be compared to a corresponding gel image. The analytical specifications of the Agilent 2100 bioanalyzer gives a sizing resolution of +/- 5%, sizing accuracy of +/- 10% and sizing reproducibility of 5% CV. We find this method to be rapid and sensitive for the detection of the UGT1A1*28 mutation.

In conclusion, UGT1A1 nt 211 G>A appears to be a risk factor for severe neonatal unconjugated hyperbilirubinemia in ethnic Malays in Malaysia. It appears to be independent of G6PD deficiency but whether it is as an added risk factor to other genetic factors remains to be studied. We have also established rapid molecular techniques that can be used for the screening of UGT1A1 mutations in neonatal jaundice.

REFERENCES

- Selvaraju S. Preliminary report: A survey on severe neonatal jaundice cases admitted to selected hospitals in Malaysia. In: Proceedings of National Perinatal Health Conference, 1999; 70-9.
- Ives NK. Gastroenterology Part 1. Neonatal jaundice. Textbook of Neonatology. 3rd ed. Churchill-Livingston, Edinburgh; 1999. 715-32 p.
- Huang MJ, Kua KE, Teng HC, Tang KS, Weng HW, Huang CS. Risk factors for severe hyperbilirubinaemia in neonates. *Pediatr Res.* 2004; 56(5): 682-9
- Aono S, Adachi Y, Uyama E, *et al.* Analysis of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. *Lancet.* 1995;345(8955): 958-9.
- Monaghan G, Ryan M, Seddon R, Hume R, Burchell B. Genetic variation in bilirubin UDP-glucuronosyltransferase gene promoter and Gilbert's syndrome. *Lancet* 1996; 347: 578-81.
- Akaba K, Kimura T, Sasaki A, *et al.* Neonatal hyperbilirubinaemia and mutation of the bilirubin uridine diphosphate-glucuronosyl transferase gene: a common missense mutation among Japanese, Koreans and Chinese. *Biochem. Mol. Biol. Int.* 1998; 46: 21-6.
- Akaba K, Kimura T, Sasaki A, *et al.* Neonatal hyperbilirubinaemia and a common mutation of the bilirubin uridine diphosphate-glucuronosyltransferase in Japanese. *J. Hum. Genet.* 1999; 44: 22-5.
- Maruo Y, Nishizawa K, Sato H, Doida Y, Shimada M. Association of neonatal hyperbilirubinaemia with bilirubin UDP-glucuronosyltransferase polymorphism. *Pediatrics.* 1999; 103: 1224-7
- Boo NY, Wong FL, Wang MK, Othman A. Homozygous variant of UGT1A1 gene mutation and severe neonatal hyperbilirubinaemia. *Pediatr Int.* 2009; 51: 488-93.
- Sutomo R, Laosombat V, Sadewa AH, *et al.* Novel missense mutation of the UGT1A1 gene in Thai siblings with Gilbert's syndrome. *Pediatr Int.* 2002; 44: 427-32.
- Wong FL, Wang MK, Boo NY, Hamidah NH, Ainoon O. Rapid detection of the UGT1A1 single nucleotide polymorphism G211A using real-time PCR with Taqman minor groove binder probes. *J Clin Lab Anal.* 2007; 21(3): 167-72.
- Yusoff S, Van Rostenberghe H, Yusoff NH, *et al.* Frequencies of A(TA)_nTAA, G71R and G493 mutations of the UGT 1A1 gene in the Malaysian population. *Biol Neonate.* 2006; 89: 171-6.
- Ainoon O, Yu YH, Amir Muhriz AL, Boo NY, Cheong SK, Hamidah NH. Glucose-6-phosphate dehydrogenase (G6PD) variants in Malaysian Malays. *Hum Mutat.* 2003; 21(1):101.
- Nopparatana C, Panich V, Saechan V, *et al.* The spectrum of beta-thalassaemia mutations in southern Thailand. *Southeast Asian J Trop Med Public Health.* 1995; 26 Suppl 1: 229-34.
- Abdullah WA, Jamaluddin NB, Kham SK, Tan JA. The spectrum of beta-thalassaemia mutations in Malays in Singapore and Kelantan. *Southeast Asian J Trop Med Public Health.* 1996; 27(1): 164-8.
- George E, Li HJ, Fei YJ, *et al.* Types of thalassaemia among patients attending a large university clinic in Kuala Lumpur, Malaysia. *Hemoglobin.* 1992; 16(1-2): 51-66.
- Sutomo R, Talib NA, Yusoff NM, *et al.* Screening for G71R mutation of the UGT1A1 gene in the Javanese-Indonesian and Malay-Malaysian populations. *Pediatrics Int.* 2004; 46: 565-9.