Rapid Detection of the UGT1A1 Single Nucleotide Polymorphism G211A Using Real-Time PCR With Taqman Minor Groove Binder Probes

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The UGT1A1 Taqman MGB probe single nucleotide polymorphism (SNP) genotyping assay was developed to detect nucleotide 211 of the UDP-glucuronosyltransferase 1A1 (UGT1A1) gene. Defects in this enzyme interfere with process of conjugation of bilirubin and cause unconjugated hyperbilirubinemia. Variation at nucleotide 211 in the coding region of the UGT1A1 gene has been shown to be prevalent in Japanese and Chinese. Using an ABI sequence detection system (SDS) 7000, an allele-specific real-time PCR-based genotyping method was established to detect nucleotide G211A. Cord blood from 125 infants without hyperbilirubinemia (controls) were compared with cord blood from 74 infants (cases) with severe hyperbilirubinemia (total serum bilirubin >300 µmol/L).

Key words: UGT1A1 gene; hyperbilirubinemia; SNP

Homozygous variation of the UGT1A1 gene at nucleotide 211(A/A) is significantly more common in cases (14.9%) than in controls (0.8%) ($P < 0.001$). Direct sequencing from 20 randomly selected samples showed eight samples with homozygous wild type, seven with homozygous variant, and five samples were heterozygous. The result from this assay was in complete concordance with the DNA sequencing result and clearly discriminate wild-type (G/G), homozygous variant (A/A), and heterozygous (G/A). This assay is rapid and robust for screening of SNP G211A to determine if this polymorphism plays a role in causing severe neonatal jaundice in the local context. *J. Clin. Lab. Anal.* 21:167–172, 2007.

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INTRODUCTION

The bilirubin uridine 5' diphosphate glucuronosyl transferase 1A1 (UGT1A1) gene is a key enzyme in bilirubin metabolism. Defects in this enzyme interfere with the process of conjugation of bilirubin and cause unconjugated hyperbilirubinemia (1). Studies have shown that bilirubin levels correlated well with the frequencies of different variants of UGT1A1 gene either in the promoter or coding region (1,2). Mutation at nucleotide 211 (G>A) in the coding region of the UGT1A1 gene, which causes Arginine to replace Glycine at position 71 (G71R) of the corresponding protein, has been shown to be prevalent in Japanese and Chinese (3,4). Various methods have been published to detect the polymorphism of the UGT gene, such as conventional PCR sequencing and PCR-restriction fragment length polymorphism (RFLP) (2–4).

We present here a another technical approach to detecting nucleotide 211 G>A polymorphism using real time PCR with Taqman minor groove binder (MGB) SNP genotyping assay (Applied Biosystems, Foster City, CA) as part of our group's effort to determine if this gene plays a role in causing severe neonatal jaundice

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in the local context. Real-time PCR is widely used for absolute or relative quantification of gene expression. In addition to quantifying gene expression, potential applications for real-time PCR include mutation detection, allelic discrimination, and detection of single nucleotide polymorphism (SNP) using the fluorogenic 5' nuclease (5). SNP detection by real-time PCR has found application in the diagnosis of human disease, pharmacogenetics, and drug development. It has replaced techniques such as sequencing, single strand conformation polymorphism, and restriction enzyme digestion.

Real-time PCR genotyping assay consists of two dye-labeled probes, one probe for each allele in the two-allele system. Each probe consists of an oligonucleotide with 5' MGB reporter dye and a 3' nonfluorescent quencher (NFQ). VIC is covalently linked to the 5' end of the probe for the detection of allele 2 (wild-type). FAM is covalently linked to the 5' end of the probe for the detection of allele 1 (mutant). Taqman MGB probes are degraded during amplification by the 5'-3' exonuclease activity of the polymerase. During degradation of the probe, the fluorescent group is separated from the quencher, leading to increased fluorescence. The fluorescence signal is normalized by dividing the emission intensity of the reporter dyes by the emission of a reference dye (Rox, 6-carboxy-X-rhodamine) included in the reaction mix. For allelic discrimination, probes matching the wild-type and mutant allele are conjugated to a different fluorescent group. Discrimination between alleles is based on the difference in melting temperature (ΔT_m) between the differently labeled match and mismatch probes.

We performed this technique on a group of Chinese neonates with severe unconjugated hyperbilirubinemia and normal infants to determine the prevalence of the various genotypes.

MATERIALS AND METHODS

Subjects

A prospective observational study was carried out on neonates from the Neonatal Intensive Care Unit (NICU) and postnatal wards, Hospital University Kebangsaan, Malaysia (UKM) from 1 April to 1 December, 2005. A total of 74 infants with severe neonatal jaundice (serum bilirubin levels $>250 \mu\text{mol/L}$ at age 1–2 days or $>300 \mu\text{mol/L}$ at age 3 days and onward) were studied. A total of 41 of the cases had known risk factors for severe hyperbilirubinemia (Table 1). G6PD deficiency is the most common cause, followed by ABO/Rh incompatibility. The remaining 33 neonates had unconjugated hyperbilirubinemia without obvious known risk factors for hyperbilirubinemia.

TABLE 1. Risk factors for neonatal hyperbilirubinemia in 74 neonates

Risk factor	n
Underfeeding/dehydration	3
ABO incompatibility	11
Rh incompatibility	1
G6PD deficiency	19
Cephalohematoma	1
Combined ABO+G6PD deficiency	6
No obvious cause	33

Infants with conjugated hyperbilirubinemia $>15\%$ of total bilirubin levels, age ≥ 28 days and premature infants <36 weeks gestation were excluded. No patients had sepsis, gastrointestinal obstruction, polycythemia, or congenital malformation. No neonate developed kernicterus in this study. The control group comprised of total 125 full-term neonates born in Hospital UKM during the study period. Parental consent was obtained for participation in the study for all 199 neonates. This project was approved by the Research and Ethics Committee, Faculty of Medicine, University Kebangsaan, Malaysia.

DNA Extraction

Ethylenediaminetetraacetic acid (EDTA) cord blood samples were sent to the Hematology Laboratory for routine screening and for DNA analysis. DNA extraction was carried out by standard techniques using Gene All (General Biosystems, Seoul, Korea).

SNP Analysis of the UGT1A1 Gene

SNP analysis for nt 211 involved the amplification of a 110-bp fragment of exon 1 of the UGT1A1 gene. Oligonucleotide primers used were designed based on the published UGT1A1 sequence (AF180372) from www.ncbi.nlm.nih.gov using Primer Express Software version 2.0 (Applied Biosystems). PCR primers and sequences are listed in Table 1. Real-time PCR was performed in a final reaction of $25 \mu\text{L}$, which contained $12.5 \mu\text{L}$ of $2 \times$ Taqman Universal Master Mix, $0.5 \mu\text{L}$ of $40 \times$ Taqman SNP genotyping assay mix (Applied Biosystems) (Table 2), and 15 ng of genomic DNA in $12 \mu\text{L}$ of distilled water. PCR amplification and detection of amplified fragment were performed on the ABI Prism 7000 SDS (Applied Biosystems). The amplification conditions were 2 min at 50°C for AmpErase uracil-N-glycosylase activity and 10 min at 95°C for Amplitaq Gold activation, followed by 40 cycles of 15 sec at 95°C for denaturation and 1 min at 60°C for annealing and extension.

TABLE 2. Primers for PCR amplification and sequencing

	Name	Sequence 5'–3'
Primer		
	Sequencing	211Fs 211Rs
	Real-time PCR	211F 211R
		TGGTTGTCCTAGCACCTGACGC CCAAAGGGAGGAGTGAAAGAG CACCTGACGCCTCGTTGTA CAGGGTACGTCTTCAAGGTGTAAA
Probe		
	Real-time PCR	211PN (VIC) 211PM (FAM)
		CATCAGAGACGGAGCAT ATCAGAGACAGAGCAT

The samples were run together with the nontemplate control in a 96-well plate. Allelic discrimination was performed on the post-PCR product. The fluorescence data of the post-PCR products were analyzed directly using allelic discrimination software of the ABI Prism 7000 instruments and presented in *xy* scatter dot plot format. Each sample was verified by examining the PCR curve generated to eliminate false-positive results due to aberrant light emission.

Sequence Analysis of *UGT1A1*

For confirmation of the real-time PCR findings, 20 samples were randomly selected and subjected to nucleotide sequence analysis for each allele by direct sequencing. The 20 samples consisted of eight samples with homozygous wild-type, seven with homozygous variant, and five heterozygous samples. Exon 1 of the *UGT1A1* gene was amplified as a single DNA fragment using primers 211Fs and 211Rs. Conditions for the PCR reaction were as follows: an initial denaturation for 5 min at 95°C followed by 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C for 35 cycles. A final extension of 5 min at 72°C was performed to ensure complete extension of PCR products. Direct sequencing of DNA was performed by the method of Yamada et al. (6) using BigDye Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA), with an Applied Biosystems 3130 genetic analyzer.

Statistical Analysis

The data was analyzed using SPSS (Chicago, IL) (Version 11.5) using Student's *t*-test for continuous variables and chi squared test for discrete variables, to compare the differences between the control group and the hyperbilirubinemic group. A *P* value of <0.05 was considered statistically significant.

RESULTS

Male/Female Ratio, Gestation Age, and Birth Weight

Table 2 shows the comparisons of male/female ratio, gestation age, and birth weight between cases and

control groups. There were no significant differences observed.

UGT1A1 Gene in Neonates

The basis of the *UGT1A1* allelic discrimination assay was the preferential degradation of the specific allele complementary to the target that was amplified. Two oligonucleotide probes, each one complementary to the wild-type (allele 2) and variant (allele 1) were mixed together in the same amplification mixture, where amplification conditions selectively digested the probe based on the presence of the allele target. After 40 cycles of PCR amplification, the fluorescence for each allele from each sample was presented in an easily interpretable graphic form (*xy* scatterplot) as shown in Fig. 1. Four clusters of points corresponding to the genotypes of GG (homozygous wild-type), GA (heterozygous), AA (homozygous variant), or no amplification were expected in the analysis. If there was fluorescent from only the reporter for the wild-type allele (VIC), the sample was genotyped as GG. If there was fluorescent from only the reporter for the mutant-type allele (FAM), the sample was genotyped as AA. If there was intermediate fluorescent from both alleles, the sample was genotyped as GA. For PCR to which no DNA was added (no template control), no fluorescence was expected except for background signal. As shown in Table 3, significant difference in the frequency of nucleotide 211G>A was found between the jaundiced neonates and the controls.

Direct Sequencing of 20 Randomly Selected Samples Found to be 100% Comparable With SNP Analysis

Figure 2A and B show the PCR amplification curve for homozygous and heterozygous samples.

Table 4 shows the distribution of *UGT1A1* genotypes and frequencies of alleles with mutated codon 71 (G71R) in the control and hyperbilirubinemic group. Table 5 shows the relationship between cases of neonatal hyperbilirubinemia of unknown causes and association with *UGT1A1* (G<A) mutation.

Fig. 1. Allelic discrimination using UGT1A1 Taqman MGB probe SNP genotyping assay. After PCR amplification, an endpoint fluorescent reading Rn (normalized reporter signal) was taken and a scatterplot of allele 2 vs. allele1 was generated using SDS software. A spot on the graph represents the reading from a well. Results are shown for the analysis of 73 samples with one nontemplate control (NTC). Results are displayed in dot-plot format. allele X; ^ allele Y; ^ allele X and allele Y; and ^ NTC.

TABLE 3. Comparison of clinical data between neonates with hyperbilirubinemia and healthy controls

	Hyperbilirubinemia group (n = 74)	Control group (n = 125)	P value
Male/female	45/29	68/57	NS
Gestation age (weeks)	38.2±1.4	38.8±1.1	NS
Birth weight (g)	3,015±386	3,064±455	NS

NS, not significant.

DISCUSSION

We used Taqman MGB SNP genotyping assay for allelic discrimination of the nucleotide 211G to A polymorphism of UGT1A1 gene in this study. To our knowledge, this is the first study using fluorescence-based SNP detection assay for UGT1A1 SNP detection. Other studies published previously had used conventional PCR sequencing and PCR-RFLP. Fluorescence-based SNP detection assays offer several important advantages over traditional PCR approaches used to determine genotype (e.g., PCR-RFLP). PCR-RFLP can in some instances result in significant false-positive results, as a result of incomplete restriction enzyme digestion or the presence of other mutations close to the mutation of interest (7,8). The use of restriction enzymes, which commonly have 4–6-bp recognition sequences, excludes the majority of available SNPs from this analysis (9). In addition, in PCR-RFLP methods, much of the time needed is devoted to postamplification steps: verifying the presence of PCR products by gel

electrophoresis, restriction endonuclease digestion, and restriction pattern visualization by gel electrophoresis. Fluorescence-based SNP detection assays are more amenable to high throughput screening, as they do not require extensive postamplification manipulation. Using this approach, genotyping results can be obtained within 2 hr. Discrimination between wild-type and mutant alleles is based on the difference in melting temperature (ΔT_m) between the differently-labeled match and mismatch probes. A 1-bp mismatch in normal Taqman probe may lead to a small T_m and subsequent difficult allelic discrimination. DNA probes conjugated to a MGB group form hyperstabilized duplexes with complementary DNA by folding of the MGB probe into the duplex, thus giving a higher T_m . The conjugation of a MGB group therefore allows the design of shorter probes (17 bp), with an increased specificity attributable to increase mismatch discrimination. To further eliminate the chance of false positives, the AmpErase system was used. This system involves the incorporation of dUTPs in place of dTTPs in all PCR products; at the beginning of each run, uracil-containing nucleic acids, including PCR products, are digested by the uracil-N-glycosylase enzyme during the 50°C step. The present assay was also designed to conform to standard thermocycling conditions (40 cycles containing a 60°C annealing and extension step), allowing the assay to be performed simultaneously with other assays, making it ideal in a clinical laboratory in which numerous assays for different SNP are being performed.

Each result was also verified by examining the PCR amplification curve generated from the software to

Fig. 2. **A:** Amplification plot for allele 2 sample. Example: Sample displays an allele 2 amplification plot, indicating a nucleotide G at position 211. **B:** Amplification plot for heterozygous sample. Example: Sample F1 displays both an amplification plot (allele 1 and allele 2), indicating both nucleotide G and nucleotide A at position 211.

TABLE 4. Distribution of *UGT1A1* genotypes and frequencies of allele with mutated codon 71 (G71R) in the control and hyperbilirubinemic groups*

	Genotype distribution					
	Codon 71 mutation (G71R)				Allele frequency	
	n	Normal	Hetero ^a	Homo ^b	n	AGA (G71R)
Control group	125	93	31	1	250	0.132
Hyperbilirubinemia group	74	45	18	11	148	0.27

*Missense mutation of codon 71, from GGA to AGA (G71R).

^aHeterozygous for AGA.

^bHomozygous for AGA, $P < 0.001$.

TABLE 5. Relationship between of unknown causes of neonatal hyperbilirubinemia and association with *UGT1A1* (G<A) mutation

<i>UGT1A1</i> mutation	n/33	(%)
Normal	19	(57.6)
Heterozygous	7	(21.2)
Homozygous	7	(21.2)

eliminate false positives due to aberrant light emission. The simple, rapid, and reproducible real-time PCR genotyping methods presented here constitute a significant improvement over current techniques.

By using this method we have successfully determined the prevalence frequency of *UGT1A1* nucleotide 211G>A polymorphism in our jaundiced neonates. We found that the prevalence of *UGT1A1* polymorphism (211G>A) in cases (39.2%) was significantly higher than controls (25.6%). Huang et al. (1) also noted that

variation at nucleotide 211 variant (44.7%) was associated with a higher percentage of jaundice cases with serum bilirubin >340 $\mu\text{mol/L}$ compared to wild-type genotypes (25.7%). Besides that, we have identified a large group of neonates jaundice, 44.6% (33/74) with no identifiable cause for severe jaundice based on clinical and laboratory data. This is similar to the previous national survey reported in which 44% of Malaysian newborn infants had no known risk factors for the development of severe neonatal jaundice (10). Our study shows that 14 out of the 33 (42.4%) neonates with hyperbilirubinemia to be due to polymorphism of the *UGT1A1* gene at nucleotide 211G>A. Homozygous 211 variant of the *UGT1A1* gene at nucleotide 211G>A (21.2%) plays a contributing factor in the development of severe neonatal jaundice in our local context. This study is an ongoing project to establish the relationship of 211G>A of the *UGT1A1* gene and severe neonatal jaundice in Malaysian Chinese newborn

infants. The use of real-time PCR with Taqman MGB probes allows for a rapid and easy method for SNP analysis of UGT1A1. A homozygous variant of the UGT1A1 gene at nucleotide 211 appears to be the risk factor for neonatal hyperbilirubinemia and accounts for 21.2% of severe neonatal jaundice in those cases with no known risk factor of hyperbilirubinemia. This assay is rapid, simple, and robust, since the instrument continuously measures fluorescence in all 96 wells of the thermal cycler during PCR amplification and the corresponding software processes and analyses the fluorescence data. We believe that this assay has a potential to become a frontline assay in the genetic screening of new born neonates.

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