

Genotyping of OATP2 Variants in a Group of Malaysian Neonates Using High-Resolution Melting Analysis

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Introduction

ORGANIC ANION TRANSPORT POLYPEPTIDE 2 (OATP2), also known as OATP-C, OATP1B1, and LST-1, is responsible for the transportation of organic anions into hepatocytes. It has been reported that OATP2 transports a broad range of endogenous and xenobiotic compounds such as bile acids, bilirubin, sulfate and glucuronide conjugates, thyroid hormones, peptides, and drugs like 3-hydroxyl-3 methylglutaryl-coenzymeA-reductase inhibitors (pravastatin, rosuvastatin, pitavastatin, and methorexate).¹⁻³ The gene for OATP2 is located at chromosome 12p12.14.1, and a number of single-nucleotide polymorphisms (SNPs) have been identified in both the encoding and regulation regions of the *OATP2* gene in different populations of the world.³ Among the Asian populations, two OATP2 variants have been shown to be highly prevalent in the Chinese in mainland China, that is, c.388A>G (73.4%) and c.521T>C (14.0%).⁴ Additionally, two other silent mutations, c.571T>C and c.597C>T of the *OATP2* gene, were also reported to be common in Asian populations, occurring at 26% and 50%, respectively, in the Chinese population,⁵ and 64.2% and 42.9% in a Japanese population.⁴⁻⁷ The importance of *OATP2* genotyping may lie in the fact that variations in the *OATP2* gene may have an impact on drug metabolism and perhaps on the pathogenesis of neonatal jaundice. One study has shown that the OATP2 c.388A>G variant may be a possible risk factor for severe neonatal hyperbilirubinemia.⁸ Malaysia is a multiethnic country comprising of major ethnic Malays, Chinese, and Indians. This study forms our initial effort to determine the existence of the *OATP2* gene variation in the Malaysian population as a prequel to study its role in cases of unconjugated hyperbilirubinemia, a common condition in Malaysian newborns. We report here our study on the determination of allelic variations in Malaysian neonates using an HRM analysis and TaqMan Minor Groove Binder (MGB) assays. Until now, these OATP2 alleles have been genotyped with either a polymerase chain reaction

(PCR)-restriction fragment length polymorphism (RFLP) assay (c.388A>G) or with an allele-specific PCR assay (c.521T>C).^{3,9} Recent study reported the use of real-time PCR with the fluorescence resonance energy transfer (FRET) assay for genotyping the c.388A>G and c.521T>C variant.¹⁰ To perform large epidemiological studies or for routine clinical use, a rapid genotype method is preferred. Therefore, the present study describes the application of HRM assays and TaqMan MGB assays. By using these methods, we were able to genotype these OATP2 polymorphisms in considerably less hands-on time and with a reduced contamination risk.

Materials and Methods

Subjects

Cord blood samples were consecutively collected from 350 full-term normal babies (206 Malays, 73 Chinese, and 71 Indians) born in the State Hospital from January 2007 till June 2007 and were subjected to the DNA analysis to determine five known OATP2 variants. Parental consents were obtained, and the project was approved by the Research Ethics Committee, Faculty of Medicine, Universiti Kebangsaan Malaysia, and the International Medical University, Malaysia.

Dried blood-spot samples collection

Five to 10 μ L of newborn neonates' blood specimen was collected on the Whatman paper (Whatman, Inc., Florham Park, NJ) and stored in a sealed multibarrier pouch (Whatman, Inc.) with one pack of desiccant (Whatman, Inc.).

Extraction of genomic DNA from dried blood spot

Four punches of 1.5-mm spot from each dried blood spot (DBS) were subjected to DNA extraction using the Qiagen

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TABLE 1. PRIMER SEQUENCES FOR HIGH-RESOLUTION MELTING ANALYSIS

<i>SLCO1B1/OATP2 SNP</i>	<i>Primer sequence</i>	<i>Fragment size</i>
A388G	F: 5'-ATCAGTGATGTTCTTACAGTTAC-3' R: 5'-CTATCTCAGGTGATGCTCTATTG-3'	133 bp
T521C	F: 5'-TTGTTTAAAGGAATCTGGGTCA-3' R: 5'-TACCTAAATACAAAGAAGAATG-3'	77 bp
Intron5 deletion	F: 5'-TGTCAAAGTTTGC AAAGTGAAT-3' R: 5'-CACATGTATGACCCAGATTCC-3'	174 bp

SNP, single-nucleotide polymorphism.

Mini Kit with the QIAcube automation system (Qiagen, Valencia, CA). Eluted DNA was then subjected to optical density (OD) reading to determine the A260/A280 ratio. Thirty nanograms of DNA from each samples was subjected to a high-resolution melting (HRM) analysis, TaqMan MGB genotyping assay (Applied Biosystems, Foster City, CA), and sequencing.

HRM analysis

HRM analysis was performed to genotype c.388A>G, c.521T>C, and IVS5-107_112 delCTTGTA (Table 1 lists the sequence of primers). Each DNA sample was diluted to a uniform DNA concentration of 15 ng/ μ L. PCRs were performed separately for each variant. For each variant, three samples of known genotypes (wild-type, homozygous mutant, and heterozygous) were included in each PCR as a reference. PCR amplification and HRM analysis were performed on the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). The 20- μ L PCR reaction consisted of 30 ng of DNA as template, 1 \times Takara Premix Buffer for Perfect Real Time, 5 μ M of SYTO 9 dye, and 150 nM of forward and reverse primers. All PCRs were performed according to the following conditions: 40 cycles of 95°C for 10 sec; 50°C for 10 sec and 72°C for 15 sec; a melt from 74°C to 84°C rising at 0.2 per second. The conditions for the variant c.521T>C were 40 cycles of 95°C for 10 sec, 56°C for 10 sec and 72°C for 15 sec, and a melt from 74°C to 84°C rising at 0.2 per second; for the variant IVS5-107_112 delCTTGTA, HRM was run in 40 cycles with denaturing at 95°C for 10 sec and 60°C for 35 sec, and a melt from 70°C to 80°C rising at 0.2 per second was done to discriminate each genotype. As a quality control measure, samples showing amplifications after 30 cycles or more, that is, the Ct value >30 (due to too little starting template amount or template degradation effects), and samples that reached the plateau phase after 35 cycles were excluded from further analysis. Melting curve data were then automatically normalized and displayed as a normalized melting curve. Normalized curves provide the basic representation of the different genotypes based on curve shifting (for homozygotes) and curve shape change (for heterozygotes). Normalized melting curves were also displayed as difference graphs, using a known sample (normally wild type) as the baseline. Genotyping for c.571T>C and c.597C>T were performed with TaqMan Genotyping assays purchased from Applied Biosystems.¹¹

Sequencing analysis

Twenty samples with various genotypes were chosen randomly and subjected to a sequencing analysis for exon 4 and

exon 5 of the *OATP2* gene to identify c.388A>G, c.521T>C, c.571T>C, c.597C>T, and IVS5-107_112. Two sets of primers were designed to amplify exon 4 and exon 5, respectively. The primers for sequencing analysis are listed as in Table 2.

Results

Genotypes for c.388A>G, c.521T>C, and IVS5-107_112 delCTTGTA were successfully discriminated by the HRM method. The HRM analysis displayed types of melting curve shapes that correlated with the different genotypes in newborn samples. For c.388A>G (Fig. 1a), the wild type (AA) showed a lower melting temperature compared to the homozygous mutant (GG), as the GC base pair is more stable than the AT base pair. The more stable homoduplex (GC base pair) was distinguished by a higher Tm than the less stable homoduplex (AT base pair). For 521T>C (Fig. 2a), the homozygous mutant genotype (CC) produced a similar curve as wild type (TT), but with a higher melting temperature, which is again consistent with the lower thermal stability of AT base pairs relative to GC base pairs. As for the third assay (Fig. 3a), samples with a IVS5-107_112 delCTTGTA deletion of 6 bp from position 63 to 68 were clearly distinctive from the wild type, as they show a lower melting temperature compared to the wild type. The melting pattern in the normalized melt curve of heterozygous variants in all three assays was clearly different from the melting patterns of homozygous samples. Samples with the homozygous deletion of IVS5-107_112 delCTTGTA showed lower melting temperatures when compared to samples without the deletion. Melting curves of the heterozygous samples showed duplex patterns. Using a previously reported TaqMan MGB assay, variants c.571T>C and c.597C>T were successfully detected.¹¹ The results of the HRM assays and TaqMan MGB assays were completely in concordance with the results of the DNA sequence analysis. The genotype frequencies of c.388A>G, c.521T>C, c.571T>C, 597C>T, and IVS5-107_112 of the *OATP-2* gene are shown in Table 3.

TABLE 2. PRIMER SEQUENCES FOR SEQUENCING

<i>Primer</i>	<i>OATP2</i>
Exon4F	5'-TCTTCTTGCTGGACACTTCC-3'
Exon4R	5'-GTGTTGTTAATGGGCGAACTG-3'
Exon5F	5'-GCAGCATAAGAATGGACTAATACAC-3'
Exon5R	5'-TACTAGATGCCAAGAATGCATGG-3'

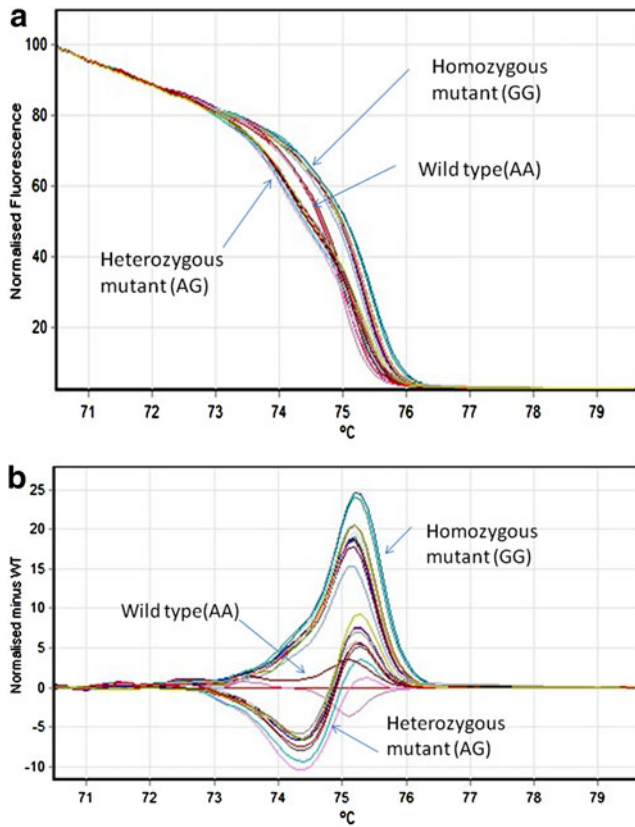


FIG. 1. (a) Normalized melt curve for c. 388A>G of OATP2 (confidence threshold, 90%; normalization region, 70.5°C–71°C and 79.1°C–79.6°C). (b) Difference graph for c. 388A>G of OATP2.

Discussion

HRM analysis distinguished the various genotypes for c.388A>G, c.521 T>C, and IVS5–107_112 delCTTGTA. Variants c.571 T>C and c.597 C>T were successfully genotyped using the TaqMan MGB assay. All samples were able to be classified accordingly from the results of the melting curve shape. Heterozygous samples were easily detected by changes of curve shape because of the greater destabilization of the two mismatches of the heteroduplexes. Samples with heterozygous genotypes showed typical heteroduplex melting patterns and were easily distinguishable from the wild-type patterns. The results of the HRM assays and TaqMan MGB assays for the three and two variants, respectively, were completely in concordance with the results of the DNA sequence analysis. New-generation intercalating dyes such as LC Green and SYTO 9 also play a role in enhancing the sensitivity and specificity genotyping of single-base changes or SNPs, especially for heterozygous mutation detection. We have shown that SYTO 9 is a suitable dye for both homozygous and heterozygous genotyping techniques for these SNPs. The combination of real-time PCR amplification and HRM analysis in Rotor Gene 6000 allows assessment of the amplification efficiency of all samples prior the HRM analysis as measurement of quality control. Normally, samples with poor or late amplification can be readily identified and excluded from further analysis, as poor-quality samples tend to give unreliable results when analyzed by HRM. The

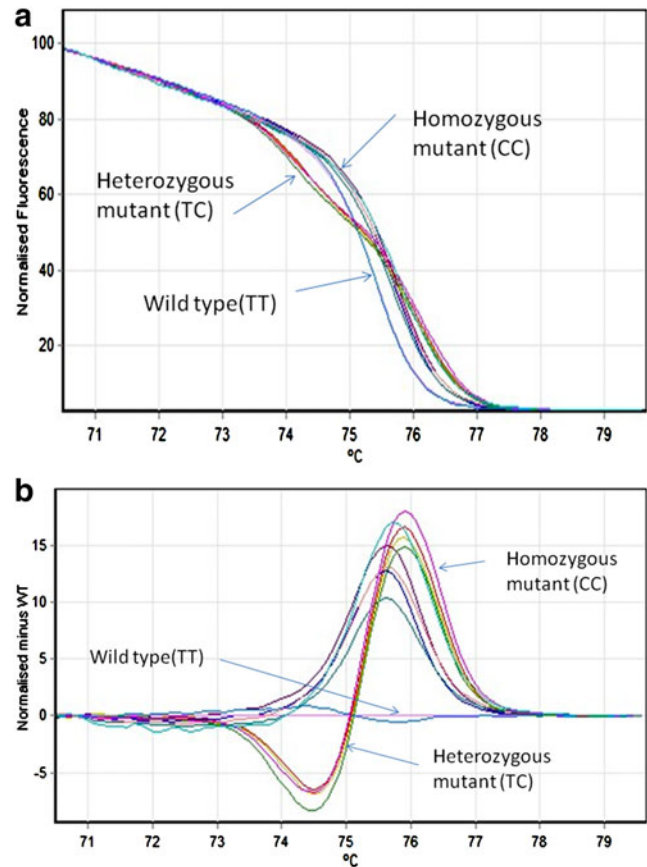


FIG. 2. (a) Normalized melt curve for c.521T>C of OATP2 (confidence threshold, 90%; normalization region, 70.5°C–71°C and 79.1°C–79.6°C). (b) Difference graph for c.521T>C of OATP2.

use of RFLP in genotyping of c.388A>G and c.521 T>C has been reported to be associated with significant unreliable results, as a result of incomplete restriction enzyme digestion or the presence of other mutations close to the mutation of interest.^{12–14} The use of allele-specific PCR relies on an SNP mismatch at the 3'-end of a primer, and can lead to unreliable

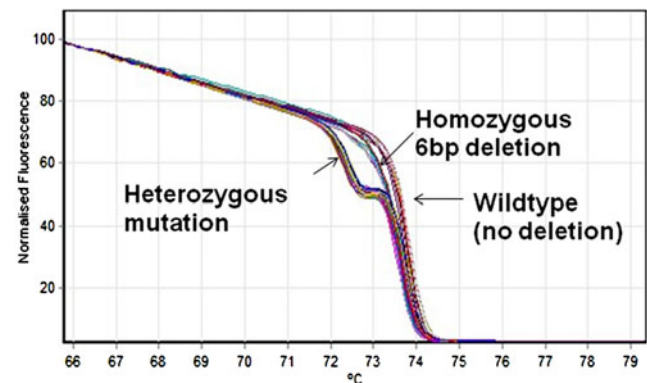


FIG. 3. Normalized melt curve for a 6-bp deletion at intron 5 OATP2 (confidence threshold, 90%; normalization region, 65.5°C–66°C and 79.0°C–79.5°C).

TABLE 3. GENOTYPE FREQUENCIES OF INDIVIDUAL SINGLE-NUCLEOTIDE POLYMORPHISM OF THE OATP2 GENE

SNP	Genotype frequency		
	Malay (n=206)	Chinese (n=73)	Indian (n=71)
c.388A>G			
AA	1.1 (14)	6.8 (5)	21.4 (16)
AG	30 (62)	27.4 (20)	44 (33)
GG	63.1 (130)	65.8 (48)	29.3 (22)
c.521T>C			
TT	72.3 (149)	78.1 (57)	59.2 (42)
TC	4.9 (10)	16.4 (12)	2.8 (2)
CC	22.8 (47)	5.5 (4)	38.0 (27)
c.571T>C			
TT	62.1 (128)	60.3 (44)	30.7 (23)
TC	29.6 (61)	15.1 (11)	37.3 (28)
CC	8.25 (17)	24.7 (18)	26.7 (20)
c.597C>T			
CC	19.9 (41)	35.6 (26)	42.7 (32)
CT	26.7 (55)	24.7 (18)	5.3 (4)
TT	53.4 (110)	39.7 (29)	46.7 (35)
IVS5-107_112 delCTTGTA			
Wildtype	12.6 (26)	27.4 (20)	20.0 (15)
Heterozygous	56.3 (116)	28.8 (21)	45.0 (34)
Homozygous	7.3 (15)	27.4 (20)	10.0 (7)

results.¹⁵ Fluorescence-based SNP detection assays such as HRM analysis and TaqMan MGB assay are more amenable to high-throughput screening, as they do not require extensive postamplification manipulation. Using these approaches, genotyping results can be obtained within 2 h. The HRM analysis method enables PCR amplification and HRM performed in a single run, and the results are available for analysis at the end of the run.¹⁶ This allows assessment of amplification for all samples before HRM analysis as a quality control measure. The use of a single instrument also minimizes the amount of manual handling, which improves turnaround times.

Recent genotyping of c.388A>G and c.521T>C using the real-time PCR FRET assay, which is based on melting temperature analysis, had shown limitation in discriminating heterozygous of c.388A>G.¹⁰ However, by using the HRM analysis, we were able to discriminate the three different genotypes easily based on the normalized melting curve. The results of the present study showed that all the five variants of the OATP2 gene are common among three major ethnic groups in Malaysia. Our findings are consistent with those of other investigators^{1,8} that c.388G>A is the most common OATP variant with a prevalence of 79.5% in the Chinese followed by 78.2% in the Malays and 54.2% in the Indians. These results confirmed the findings by others that c.388A>G is common in the Asian populations. The Indians showed the highest frequency (38%) for c.521T>C followed by the Malays (22.8%), whereas the frequency is low among the Chinese (5.5%). The allelic frequency for c.571T>C is highest in the Indians (47.9%), followed by Chinese (32.1%) and Malays (23.1%). The allelic frequency of c.597C>T (ranging from 52% to 66.7%) is in line with other published study.^{5,9} The variations of c.571C>T and c.597T>C are silent mutations and

are generally disregarded. However, a study by a Japanese group showed that silent mutations were able to change the rate of protein folding, and thus affect the affinity of the protein toward substrate binding.¹⁷ The IVS5-107_112 6-bp deletion at the intron 5 of OATP2 is a new finding in our population. The allelic frequency of this deletion was shown to be 40% in the Japanese population.¹⁸ We found 50% of Chinese harboring this deletion, 46.5% in the Malays, and 42.9% in the Indians, suggesting that it is a common variation in our population.

Conclusion

HRM and MGB probe are two techniques, which are rapid, sensitive, and specific methods, for screening of OATP2 gene variations in clinical samples and represent a significant advance in techniques for mutation detection. Future studies are planned to investigate the impact of various haplotypes of OATP2 in our population, as this may contribute to individual personalized drug therapeutics and pathogenesis of hereditary diseases.

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Author Disclosure Statement

No competing financial interests exist.

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