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Rapid identification of CYP2C8 polymorphisms by high resolution melting analysis

Chun-Chi Chang [a,b], Pei-Chin Lin [a,c], Ching-Hsiung Lin [a,b], Kun-Tu Yeh [a,d], Hsiao-Yu Hung [e], Jan-Gowth Chang [a,e,f,g,*]

[a] Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Division of Chest Medicine, Department of Internal Medicine, Changhua Christian Hospital, Changhua, Taiwan
[c] Division of Hematology and Oncology, Department of Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[d] Department of Pathology, Changhua Christian Hospital, Changhua, Taiwan
[e] Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[f] Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[g] Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

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ABSTRACT

Background: Cytochrome P450 (CYP) 2C8 is the principal enzyme responsible for the metabolism of arachidonic acid and various drugs, and influences drug-drug interactions and some associated diseases. Large interindividual differences in CYP2C8 enzymatic activity and several nonsynonymous genetic variations have been reported in different races. Therefore, how to identify CYP2C8 polymorphisms efficiently for genotyping in different populations is very important.
Methods: A high resolution melting (HRM) analysis was used to characterize the CYP2C8 polymorphism. Genomic DNA was extracted from peripheral blood samples from 95 normal individuals in Taiwan. Nine exons of the CYP2C8 gene were screened by HRM analysis. All results were confirmed by direct DNA sequencing.
Results: Five new single nucleotide polymorphisms (SNPs) were found in this study; two SNPs [1189 G>A (D397N) and 1230 C>T (G410G)] were in exon 8 and the others [1312 G>C (E438Q), 1497 T>C (A499A) and 1677delT (559delL)] were in exon 9. The 1497 T>C (A499A) was the most common variant with an allele frequency of 20.53% but without amino acid substitution.
Conclusions: HRM analysis is a fast, reliable, accurate and cost-effective screening method for gene mutations, even very similar cDNA sequences with 83% identities, compared with CYP2C8 and CYP2C9.

1. Introduction

Cytochrome P450 (CYP) enzymes play an important role in the metabolism of many exogenous carcinogens and endogenous substrates [1]. CYP2C8 (Cytochrome P450, family 2, subfamily C, polypeptide 8), a member of the CYP450 mixed-function oxidase system, is involved in the metabolism of xenobiotics in the body, including the anticonvulsive drug mephenytoin, benzo(a)pyrene, 7-ethyoxycoumarin, and the anticancer drug taxol. This protein localizes to the endoplasmic reticulum and its expression is induced by phenobarbital. Expression of CYPs can be induced by their substrates and has been shown in many tissues, including liver, lung tissue, intestine, and others [2–4]. Because the CYP epoxygenase pathway produces arachidonic acid metabolites that are vasoactive and affect renal sodium handling, the associated disease relevance of CYP2C8 includes hypertension [5], cachexia [6], myocardial infarction [7], cancers [8], etc.

CYP2C8 contains nine exons and is located within a cluster of CYP450 genes on chromosome 10q24. Several transcript variants encoding a few different isoforms have been found for this gene. The cDNA sequence of CYP2C8 has 1866 bp and that of CYP2C9 has 1845 bp. Because they have very similar cDNA sequences with 83% identities and 4% gaps, it is very difficult to specify CYP2C8 only. Techniques to identify unknown polymorphisms include direct sequencing, electrophoretic mobility assays, microarray, cleavage/ligation, etc. [9] Direct sequencing can detect any type of unknown polymorphism and its position, but its major limitations are that it is difficult to detect low-level mutations and it may have multiple reactions for large genes. The other techniques also have some limitations unique to the underlying technology, including that they do not locate the position of the polymorphism or they have higher false positive rates. To resolve the above major limitations, high resolution melting (HRM) analysis confirmed by direct sequencing over the range of codons of interests is a rapid, accurate and low-cost technology for mutation scanning and genotyping [10–18].

* Corresponding author at: Department of Laboratory Medicine, Kaohsiung Medical University Hospital, No. 100 Shih-Chuan 1st Rd., Kaohsiung, Taiwan. Tel.: +886 7 3115104; fax: +886 7 3213931.
E-mail address: jgchang@ms.kmuh.org.tw (J.-G. Chang).

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Evaluation of mutation scanning by HRM analysis has shown high sensitivity and specificity, concluding that HRM in the presence of a saturating dsDNA binding dye is a suitable and specific technique for mutation scanning. It is a closed-tube method, indicating that polymerase chain reaction (PCR) amplification and subsequent analysis are sequentially performed in the well, making it more convenient than other scanning methodologies. The aim of this study was to assess the value of the HRM analysis by using real-time PCR to identify CYP2C8 polymorphisms confirmed by direct sequencing over the range of codons of interests.

2. Materials and methods

2.1. Patient samples and DNA extraction

Genomic DNA samples extracted from peripheral whole blood were obtained from 96 normal individuals on health examination at Kaohsiung Medical University Hospital. The purified blood granulocytes were separated by density-gradient centrifugation using Ficoll-Paque PLUS (GE healthcare Bio-Sciences AB, Uppsala, Sweden). Subsequently, the DNAs of the blood granulocytes were extracted using the NucleoSpin® Blood Kit (Macherey-Nagel) according to the manufacturer's instructions. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital.

2.2. Primers design and PCR conditions

Good amplicon design is essential to obtain robust and reproducible HRM analysis. Generally, it is recommended to use PCR products smaller than 250 bp for best results. The difference between the melting curves of the wild-type and the heterozygote DNAs becomes smaller and more difficult to differentiate if the product length increases [12]. In this study, we designed the primer sets on the CYP2C8 DNA sequences (NCBI Reference Sequence: NG_007972.1). Table 1 shows the primer sets for the detection of CYP2C8 gene mutations in exon 1 to exon 9. All the primers synthesized were of standard molecular biology quality (Protech Technology Enterprise Co., Ltd, Taiwan). Extra care needs to be exercised in designing PCR reactions to avoid primer dimers and nonspecific amplification in HRM analysis.

2.3. HRM techniques

Determination of mutations in the CYP2C8 gene was performed by HRM analysis. The LightCycler® 480 High Resolution Melting Master kit (Roche) was used according to the manufacture's manual, and the data was subsequently analyzed using LightCycler® 480 Gene-Scanning Software ver. 1.0 (Roche Diagnostics).

PCR reactions were carried out in duplicate in 20 μl of final volume using the LightCycler ® 480 High-Resolution Melting Master (Reference 04909631001, Roche Diagnostics) 1× buffer, containing Taq polymerase, nucleotides and the dye ResoLight, and 30 ng DNA. The primers and $MgCl_2$ were used at a concentration of 0.25 μmol/l and 2.5 mmol/l, respectively, to detect the CYP2C8 polymorphism.

The PCR program required a SYBR Green I filter (533 nm), and it consisted of an initial denaturation–activation step at 95 °C for 10 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 58 °C 15 s and elongation at 72 °C for 15 s with reading of the fluorescence; acquisition mode: single). The melting program included three steps: denaturalization at 95 °C for 1 min, renaturation at 40 °C for 1 min and subsequent melting that consisted of a continuous reading of fluorescence from 60 °C to 90 °C at the rate of 25 acquisitions per °C. Curves for each duplicate were checked on the shape and peak height to meet reproducibility.

2.4. Direct DNA sequencing

Normalized and temperature-shifted melting curves from HRM, suggestive of single nucleotide polymorphisms (SNP), were distinguished and the samples were subjected to direct sequencing using the same sense and anti-sense primers in HRM analysis as described previously [11,13,14]. After HRM analysis, the samples were purified using the PCR-MTM clean-up system (VIOGEN, Sunnyvale, CA, USA). The PCR products generated after HRM were sequenced directly. The sequence reaction was performed in a final volume of 10 μl, including 1 μl of the purified PCR product, 2.5 μmol/l of one of the PCR primers, 2 μl of the ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems) and 2 μl 5× sequence buffer. The sequencing program started from 96 °C for 1 min and then was followed by a 25-cycle PCR program (denaturation 96 °C 10 s; annealing 50 °C 5 s and elongation 60 °C 4 min). The sequence detection was performed in the ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to standard protocols.

3. Results

3.1. Screening of the CYP2C8 gene mutations in the exon 1 to exon 7 regions

To identify CYP2C8 gene mutations in the exon 1 to exon 7 regions, we designed primer pairs specifically for every exon region, as shown in Table 1. During the screening process in this section, we found it was difficult to specify exon 4 using only one or two sets of primer pairs because of the very similar cDNA sequences in the other CYP450 subfamily, especially CYP2C9. To resolve the

Table 1
Primer pairs for CYP2C8 HRM and sequencing.

Primer pairs for amplified and sequenced regions		Forward primer	Reverse primer
		Sequence (5′–3′)	Sequence (5′–3′)
Exon 1	P1-1	GGCCAAAGTCCACTCAGAAA	GGAGGGAGCTTCCTTCTCCT
	P1-2	TGCAAGCTCACAGCTGTCTT	CCTTTGCAATTGGCTGGAG
Exon 2		TTTGGAGCAAATAGCGACTTA	CCTCACCCCAGTTACCAAAG
Exon 3		TGGCTGTGAATTCTCCCAGT	TGAAGACAGGTAACTGTTAAGGTCA
Exon 4	P4-1	TCCTTTATGTCTTAACAAATGCAAA	AACGGAGCAGATCACATTGC
	P4-2	AGCTTCACCCTGTGATCCCA	ATCCTGAAGTTTTCATTGAATC
	P4-3	CGTTGTTTTCCAGAAACGAT	CAAGTCTTCCCTACAACCTTGA
Exon 5		TTTCAATCAGGGCTTGGTGT	TCACAAAATGGACAAGAAATCAA
Exon 6		AGTGTATTTAGATATACTGGCA	TCAGCATTGTTCTGAAATTCACT
Exon 7		AATGTTCCATATATCTTCGTTTCC	GCACTATGGAAATTTCAGAAGTACAG
Exon 8		TCTCCTCACTTCTGGACTTCTTT	TCCAAAAAGTTCTCTCTTTCCTTT
Exon 9	P9-1	CACAGTCACAGTTAAACACAAGGA	TCAGACAGGGATGAAGCAGA
	P9-2	CTCAATACTACTGCAGTTACC	GGAAGATTTGATGAGAGGTCAG
	P9-3	CGATCTGCTATCACCTGCAA	CATTCACAAAAGAAATGGATCTAAA
	P9-4	TTGCTGCATATGCTAATACTTTTC	GCCTCCTGGCACTCTCTTTT