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Robust *CYP2D6* genotype assay including copy number variation using multiplex single-base extension for Asian populations

Eun-Young Kim [a,1], Sang-Seop Lee [b,1], Hyun-Ju Jung [b], Hye-Eun Jung [b], Chang-Woo Yeo [b], Ji-Hong Shon [a,b], Jae-Gook Shin [a,b,*]

[a] Department of Clinical Pharmacology, Inje University Busan Paik Hospital, Busan, Republic of Korea
[b] Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, Busan, Republic of Korea

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ABSTRACT

Background: We developed a *CYP2D6* genotyping method that includes copy number variation (CNV) and recently known functional haplotypes using multiplex single-base extension (SBE).
Methods: Twelve *CYP2D6* alleles (**1*, **2*, **5*, **10*, **14*, **18*, **21*, **41*, **49*, **52*, **60*, and a duplication of *CYP2D6*) were genotyped using 2 PCR reactions followed by multiplex SBE with 10 primers and singleplex SBE with 1 primer. The result from 758 Korean samples was validated by comparison with the results of direct sequencing or other genotyping methods. We also genotyped 89 Chinese and 122 Vietnamese subjects to determine the presence of recently identified functional alleles.
Results: All 12 *CYP2D6* alleles, including gene deletion and duplication, were obviously discriminated. The concordance rate was 100% between our method and other methods. Our method also covered over 98% of the *CYP2D6* genotypes in Japanese and Chinese subjects based on reported data. In addition to published genotypes, **14*, **21*, **41*, **49*, and **52* were found in about 5% in Chinese and Vietnamese.
Conclusions: The *CYP2D6* genotyping method may be clinically applicable for Asian populations. The method can be improved easily to cover other ethnic groups by utilizing additional haplotype tagging SNPs.

1. Introduction

Inter-individual genetic variation in drug responses may cause therapeutic failure or toxicity [1,2]. Cytochrome P450 (CYP) super family consists of >50 enzymes such as CYP2C9, CYP2C19, CYP2D6, and CYP3A4 [3]. They metabolize many endogenous compounds, drugs, and xenobiotics. CYP2D6 (GenBank accession number M33388) is involved in metabolizing >100 drugs, including those that have relatively narrow therapeutic ranges, such as antipsychotic, antidepressant, and antiarrhythmic agents [4,5]. It is also one of the most genetically variable of the CYPs, with 78 currently identified allelic variants [6]. In addition, there are unique ethnic differences in *CYP2D6* genetic variance, which lead to differences in patterns of drug metabolism according to ethnicity [7]. Individuals are classified as ultra-rapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs) according to their enzymatic metabolic ability [8]. Therefore, the drug metabolizing ability determined by the genotype may affect the success or failure of therapy.

Although useful clinically, available methods used for *CYP2D6* genotyping, such as PCR-RFLP, AS-PCR, sequencing, and microarray technology, such as AmpliChip® and DrugMEt™, are both costly and time-consuming to perform. Therefore, it is necessary to develop a new method that will allow the testing of all the important functional genetic polymorphisms of *CYP2D6* simultaneously and rapidly in a cost-effective manner. A method involving multiplex primer extension reaction, proposed by Sistonen et al. [9], has been developed to overcome these problems. However, this method does not include the detection of copy number variation (CNV), such as duplication and deletion, and cannot be applied to Asian populations, including Koreans, as a result of ethnic differences. Therefore, we have developed additional techniques to perform *CYP2D6* genotyping rapidly and simultaneously, to detect significant polymorphisms including CNV in Koreans and other Asians, using a multiplex single-base extension (SBE) assay. In this genotyping method, we also included recently identified functional alleles [10].

2. Materials and methods

Haplotype tagging SNP (htSNP) combinations were selected using SNPtagger [11], which covers most of the Korean haplotypes, based on our previous frequency results in 758 healthy volunteers [10]. CYP2D6 genotype to phenotype relationship was also evaluated in the previous study. All volunteers gave informed consent to participate

* Corresponding author. Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, 633-165 Gaegum-dong, Jin-gu, Busan 614-735, Republic of Korea. Tel.: +82 51 890 6709; fax: +82 51 893 1232.
E-mail address: phshinjg@inje.ac.kr (J.-G. Shin).
[1] These authors contributed equally to this work.

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in the study which was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea). Molecular haplotyping was performed to determine the actual haplotypes of the unknown gametic phase for the selection of the htSNPs. Twelve haplotypes were targeted for the genotype: **1*, **2*, **10*, **14*, **18*, **21*, **41*, **49*, **52*, **60*, duplication (**1xN*, **2xN*, **10xN*), and deletion (**5*) of *CYP2D6*. Among them **1* was determined when the screened alleles were not detected. The best tagging SNP combination among the haplotype SNP combinations was chosen after checking the overlap pattern between diplotypes in the multiplex SBE assay. To check the duplication haplotype, a singleplex SBE assay was designed separately, which could not be performed in the multiplex SBE assay because the primers of the duplication and deletion genes were of the same sequence (Table 1). A diagram to indicate the positions of long PCR primers and multiplex and singleplex SBE primers on *CYP2D6* gene is shown on Fig. 1.

The entire *CYP2D6* gene, including the nine exons and 1650 bp of the 5′-UTR, was amplified by long and accurate PCR (LA-PCR) (TaKaRa LA-PCR kit; TaKaRa Shuzo, Shiga, Japan) using the primers, CYP2D6-4F (5′-GTA TCA GGT AGT CAC AGT GGC TC-3′) and 3′2D6 (5′-ACT GAG CCC TGG GAG GTA GGT A-3′) [12]. Genomic DNA (60–120 ng) was dissolved in LA-PCR buffer containing 2.5 mmol/L $MgCl_2$, 0.4 μmol/L primers, 1.6 mmol/L dNTPs, and 1 U LA-*Taq* polymerase. PCR was performed in a volume of 20 μL. Initial denaturation at 94 °C for 1 min was followed by 30 cycles of 98 °C for 20 s, 64 °C for 30 s, and 72 °C for 7 min, with a final elongation step at 72 °C for 10 min.

To detect deletion and duplication of the entire *CYP2D6* gene, we performed two long PCR reactions. The duplicated alleles of *CYP2D6* were amplified using the primers, Dup-F_2 (5′-CCT CAC CAC AGG ACT GGC CAC C-3′) and Dup_R (5′-CAC GTG CAG GGC ACC TAG AT-3′), for amplifying a 2.8-kb direct repeat element (CYP-REP) located in the *CYP2D6–CYP2D6* intergenic region (Fig. 1) [13,14]. CYP-REP in *CYP2D7* region is also amplified using the primers (Fig. 2). The resulting amplicon of *CYP2D6*, 3238 bp in length, was genotyped for the presence of a duplication-specific SNP (Fig. 2). For the entire deletion, the *CYP2D6* gene was amplified using the primers, CYP2D6_3 (5′-ACC TCT CTG GGC CCT CAG GGA-3′) and 3′2D6*5 (5′-CAG GCA TGA GCT AAG GCA CCC AGA C-3′) [12]. The PCR products include the amplification of CYP-REP in the deletion allele but also CYP-REP in the *CYP2D6* region (Fig. 2). The resulting amplicon was analyzed for the presence of *CYP2D6*5*-specific SNP (Table 1). Both *CYP2D6* duplication and deletion alleles can be discriminated by the allele-specific SNPs located within the recombinant regions (Table 1, Fig. 2). The genomic DNA (40–80 ng) was dissolved in LA-PCR buffer containing 2.5 mmol/L $MgCl_2$, 0.4 μmol/L primers, 1.6 mmol/L dNTPs, and 1 U LA-Taq polymerase in a volume of 20 μL. PCR was performed using a 9700 Thermal Cycler (PE Applied Biosystems, Foster City, CA) with the following conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles of 98 °C for 20 s, 64 °C for 30 s, 72 °C for 3 min 30 s, and a final elongation step at 72 °C for 10 min. The PCR products were treated with 2 μL of ExoSAP-IT® per 5 μL of PCR product at 37 °C for 30 min, and then incubated at 80 °C for 15 min to inactivate the enzyme.

The pooled PCR product of the entire and deletion genes purified by ExoSAP-IT® was used as a template to detect ten polymorphic positions of the *CYP2D6* gene (Table 1). The primer lengths were designed to avoid overlapping peak signals (Table 1) with the nucleotide difference between primer lengths, about five. The final length was achieved by the addition of 5′ poly (dT) tails to the primers. The PCR product of the duplication gene purified by ExoSAP-IT® was used as a template to detect the duplication gene in the singleplex SBE (Table 1).

Multiplex and singleplex SBE were performed using SNaPshot® in accordance with the manufacturer's protocol based on annealing of a single fluorescently labeled dideoxynucleotide triphosphate on unlabeled oligonucleotide primers to detect SNPs as showing four different colors according to a nucleotide (Applied Biosystems). Briefly, 3-μL aliquots of purified PCR product were mixed with 1 μL of SNaPshot Multiplex Ready Reaction Mix, 4 μL of half-term buffer (200 mmol/L Tris–HCl, 5 mmol/L $MgCl_2$, pH 9), and pooled detection primers for multiplex SBE or a primer for singleplex SBE (Table 1) in a final volume of 10 μL. The reaction consisted of 40 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s.

Samples were treated with 1 U of shrimp alkaline phosphatase at 37 °C for 1 h, and then incubated at 65 °C for 15 min to inactivate the enzyme. The SNaPshot products were finally analyzed by capillary electrophoresis, for which 0.5 μL of the SNaPshot product was mixed with 9.3 μL of Hi-Di formamide and 0.2 μL of Genescan-120 LIZ™ size marker. The samples were denatured at 95 °C for 5 min and run on an ABI-Prism 3100 genetic analyzer using a 36-cm capillary array and POP-7 polymer. Analyses were performed with GeneMapper (ver. 3.7) software.

We tested the origin of the duplication according to a published method [15] if there were two peaks in the SNaPshot duplication test and the genotype consisted of two duplication candidates, a combination of **1*, **2*, and **10*. We mainly focused on **1*, **2*, and **10* for duplication allele detection based on our previous results.

The 758 samples from our published *CYP2D6* study [10] were used for genotyping using our method. For validation, 56 of the DNA samples were analyzed by direct sequencing of the full-length *CYP2D6* gene [10] to compare with this new method. The rest were performed as described in the previous paper [10].

To genotype 89 Chinese and 122 Vietnamese, DNA samples stored in the DNA Repository of the Pharmacogenomics Research Center (PGRC), Inje University, were used [16–18]. The racial background of the Chinese subjects was Han. All 122 Vietnamese were Viet Kinh, a

Table 1
Primers used in the multiplex and singleplex SBE[a] methods for detecting *CYP2D6* alleles.

Primer	Sequence	Concentration in the SNaPshot reaction, μmol/L	Detected polymorphism	Related *CYP2D6* allele(s)
2D6 + 1758F(P19)	CGCCTTCGCCAACCACTCC	0.05	1758G>A	**14*
2D6 + 100F(P21)	CAACGCTGGGCTGCACGCTAC	0.2	100C>T	**10*, **49*, **52*
2D6 + 1611R(P30)	$(T)_{10}$GGGCCCATAGCGCGCCAGGA	0.3	1611T>A	**49*
2D6 + 2573F(P38)	$(T)_{17}$GGGACCCAGCCCAGCCCCCCC	0.02	2573_2574insC	**21*
2D6 + 2988F(P39)	$(T)_{20}$AGTGCAGGGGCCGAGGGAG	0.3	2988G>A	**41*
2D6 + 3877F(P45)	$(T)_{25}$CTGGGCATCCAGGAAGTGTT	0.3	3877G>A	**52*
2D6 + 4125F(P50)	$(T)_{30}$CAGCTTCTCGGTGCCCACTG	0.04	4125_4133dupGTGCCCACT	**18*
2D6 + 2850R(P55)	$(T)_{35}$CAGGTCAGCCACCACTATGC	0.06	2850C>T	**2*, **14*, **21*, **41*
2D6 + 1887R(P60)	$(T)_{40}$AGGGAGGCGATCACGTTGCT	0.2	1887_1888insTA	**60*
2D6-5R(P65)	$(T)_{45}$CTCGTCACTGGTCAGGGGTC	0.05	*CYP2D6* deleted	**5*
CYP2D6-5R(P20)[a]	CTCGTCACTGGTCAGGGGTC	0.2	*CYP2D6* duplicated	**1xN*, **2xN*, **10xN*

The primer length is described within parenthesis after P. F and R in the end of a primer name mean forward and reverse primers, respectively.
[a] The SNaPshot reaction for detecting duplication in a separate well.