Drug Metab. Pharmacokinet. 29 (3): 283–285 (2014).

Note

Long PCR-based Genotyping for a Deleted *CYP2D6* Gene without DNA Extraction

Tomoko Ota, Mariko Hayashida, Minori Ishii, Kyoko Iwao-Koizumi, Shigenori Murata and Kenji Kinoshita*

School of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Japan

Full text and Supplementary materials of this paper are available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: In the post-genome era, a simple and inexpensive method for diagnostic analysis is in high demand. Cytochrome P450 (CYP) 2D6 is one of the most widely investigated CYPs in relation to genetic polymorphism. Detection of *CYP2D6*5* is difficult since long PCR is used. Especially for samples without DNA extraction, the detection is not sensitive enough for population analysis. Therefore, we developed a *CYP2D6*5* genotyping method that involves nested long PCR, directly using human whole saliva as a template without DNA extraction. This method will be very useful for genetic diagnoses and can be an efficient tool for individualization of drug therapy in clinical studies.

Keywords: cytochrome P450 2D6; *CYP2D6*5*; nested PCR; dried saliva

Introduction

In general, it is well known that genetic polymorphisms are involved in inter-individual variations in the metabolism of numerous drugs in humans.[1)] Mutations in a gene coding for a drug-metabolizing enzyme cause enzyme variants with high, low or no activity. Polymorphisms can be classified into three main phenotypes: poor metabolizers (PM), intermediate metabolizers (IM), and rapid metabolizers (RM).[2)] The PM condition may lead to an excessive or prolonged therapeutic effect or drug-related toxicity after a normal dose, conferring a genetic predisposition to drug-induced adverse effects. In the case of compounds that need to be activated by drug metabolizing enzymes, however, the PM condition may result in decreased response. On the other hand, RMs may not achieve therapeutic levels of the drug given at a standard dose and this might account for a lack of therapeutic effect.

CYP2D6 has received significant attention since the beginning of the 1970s.[3)] One of the reasons for the significant research interest in this enzyme is the wide inter-individual variation in the enzyme activity of CYP2D, which led to the discovery of deletion and duplication of the *CYP2D6* gene. CYP2D6 plays an important role in the metabolism of at least 25% of current therapeutic drugs;[3)] for example, typical substrates for CYP2D6 are largely lipophilic in nature and include tamoxifen,[4–6)] dextromethorphan,[7–9)] codeine[10,11)] and numerous other drugs. *CYP2D6*5* causes a defect in enzyme activity, which is one of the most important *CYP2D6* polymorphisms in clinical studies on Japanese subjects.[9)]

In the former long PCR assay for *CYP2D6*5*,[12)] misinterpretation could occur if the long PCR failed or if insufficient genomic DNA was added. These failures are avoided in the long PCR by inclusion of the simultaneous amplification of the cloned *CYP2D6* as an internal control for the reliability of the PCR. It can prevent misinterpretation without the need for internal control to be performed by using the multiplex primers.

Presented here is a specific example of the nested long PCR which is based on the deletion of *CYP2D6* for genotyping performed directly using dried saliva on filter paper without DNA extraction. In previous reports, *CYP2D6*5* could be detected using an extract sample from whole blood.[9,12,13)]

The aim of the present study is to demonstrate an inexpensive and high-throughput genotyping method that detects *CYP2D6*5* by nested long PCR assay using dried whole saliva without DNA extraction. Direct use of dried saliva on filter paper considerably decreases the possibility of contamination between samples.

Materials and Methods

Human genomic DNA samples: *CYP2D6* genotyping was based on long PCR using dried whole saliva disks. Samples for genotype distribution were taken from 46 healthy Japanese volunteers whose saliva had been analyzed by the long PCR method. A few drops of saliva were applied to the filter paper and dried for one hour at room temperature. The local ethics committee approved this study, and informed consent was obtained from each participating volunteer.

Detection of the *CYP2D6*5* genotyping by nested long PCR assay: The first-round long PCR assay was performed with a 50 μL reaction volume containing 5.0 μL of distilled water, 25 μL

Received October 21, 2013; Accepted December 14, 2013
J-STAGE Advance Published Date: December 31, 2013, doi:10.2133/dmpk.DMPK-13-NT-116
**To whom correspondence should be addressed:* Kenji Kinoshita, Ph.D., *School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya 663-8179, Japan. Tel. +81-798-45-9982, Fax. +81-798-41-2792, E-mail: kenji_k@mukogawa-u.ac.jp*

of 2× PCR buffer for KOD FX Neo, 10 µL of 2 mM dNTPs, 15 pmol each of CYP2D6_3 and CYP2D6_4 primers, 30 pmol each of CYP2D6_1 and CYP2D6_2 primers, and 1 U of KOD FX Neo DNA polymerase (1 U/µL, KFX-201, Toyobo, Osaka, Japan). The dried saliva was punched with a 2 mm diameter disk and put into the reaction mixture directly without DNA extraction. PCR primers, shown in **Supplementary Table 1**, were arranged from the previous report.[13,14] The amplification conditions for the first-round PCR reaction were as follows: 95°C for 5 min, followed by 5 cycles of 98°C for 10 s and 74°C for 10 min, followed by 5 cycles of 98°C for 10 s and 72°C for 10 min, followed by 5 cycles of 98°C for 10 s and 70°C for 10 min, followed by 5 cycles of 98°C for 10 s and 68°C for 10 min, followed by 20 cycles of 98°C for 10 s, 65°C for 30 s and 68°C for 10 min and a final elongation step of 68°C for 10 min. The amplification conditions were arranged from the step-down long PCR assay conditions in the Toyobo protocol.

Subsequently, 1 µL of first-round PCR product was subjected to nested PCR in a 50 µL mixture, containing 4.0 µL of distilled water, 25 µL of 2× PCR buffer for KOD FX Neo, 10 µL of 2 mM dNTPs, 15 pmol each of CYP2D6_3 and CYP2D6_7 primers, 30 pmol each of CYP2D6_5 and CYP2D6_6 primers, and 1 U of KOD FX Neo DNA polymerase. The amplification conditions for the nested PCR assay were the same as for detection of the first-round PCR reaction. The resulting PCR products were separated and detected by electrophoresis in 1% agarose gel.

Direct sequencing analysis: After confirmation of the PCR products by the nested long PCR assay, we cut out the gel and purified it using a Takara SUPREC®-EZ kit (Takara Bio, Otsu, Japan). Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit ver.1.1 (Applied Biosystems, Foster City, CA). ABI PRISM310 Genetic Analyzer (Applied Biosystems) was used for sequencing.

Results and Discussion

The gene controlling the cytochrome P450 2D6 protein, *CYP2D6*, is located on the long arm of chromosome 22. A pseudo-gene *CYP2D8P* and a related gene *CYP2D7* are located upstream from *CYP2D6*. For the sequence specificity, the extended primers of several nucleotides to 3′ downstream from the previous report[13,14] were used for the first-round PCR. These specimens in **Figure 1** were used to optimize the experimental conditions of *CYP2D6*5* genotyping. As expected, two bands of 5.1 kb and 3.2 kb were amplified using primers CYP2D6_1, CYP2D6_2, CYP2D6_3 and CYP2D6_4, as shown in **Figure 1A**. After the first-round PCR, it was possible to amplify and view three of the seven specimens in the gel in **Figure 1A**. For the nested PCR assay, we designed nested primer sets inside of the first-round PCR primers. Since there are intricate repetitive sequences that include 72 bp, 184 bp and 2,526 bp, shown as a combination of α, β and γ in **Figure 1B**, respectively, it was necessary to avoid these sequences. Compared to the previous report,[13] it was possible to shorten products even more with primers within 1 kb and 150 bp for wild-type and deleted genes, respectively. As shown in **Figure 1B**, 4.1 kb and 3.1 kb products were amplified using CYP2D6_3, CYP2D6_5, CYP2D6_6 and CYP2D6_7 primers. All genotypes of *CYP2D6*5* could be determined after the nested PCR.

The result of the experiment demonstrated that direct PCR amplification using dried saliva as a template could be successfully performed in long PCR. As in our previous report,[15] the dried saliva samples were stable for several weeks at least.

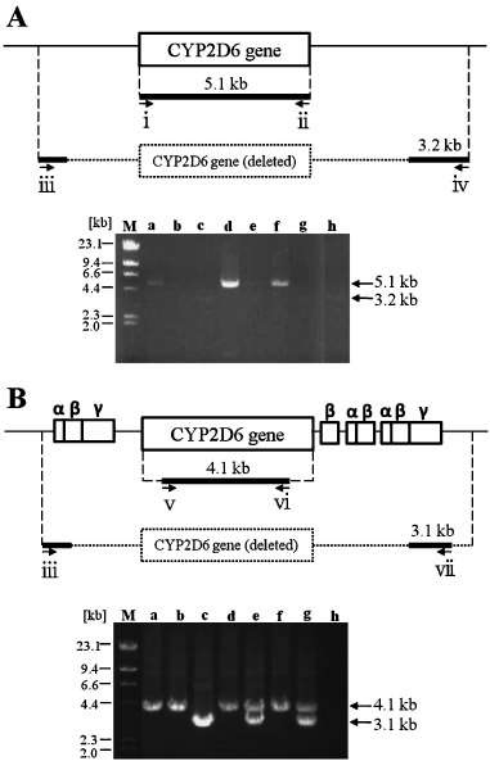

Fig. 1. Scheme of long PCR analyses for *CYP2D6*5*
(A) The first-round PCR was performed with four primers, i–iv, as indicated in the upper panel. The dotted line indicates deletion of *CYP2D6*5*. Samples were electrophoresed in 1% agarose gels in 0.5× Tris-acetate-EDTA buffer. Two bands, 5.1 kb and 3.2 kb in the gel, were amplified as the result of the first-round long PCR. (B) The nested PCR was performed with four primers, iii and v–vii. There are intricate repetitive sequences around the *CYP2D6* gene which are indicated by α, β and γ. Two bands of 4.1 kb and 3.1 kb products were amplified. The detection of *CYP2D6*5* is as follows. Lane a, b, d and f: **1/*1*, Lane c: **5/*5*, Lane e and g: **1/*5*, Lane h: negative control. M is the size standard marker of *λ-Hin*dIII digest.

Table 1. Genotype distribution of *CYP2D6*5* in 46 healthy Japanese volunteers (%)

Genotype	This study	Japanese[8]
*1/*1	82.6	87.7
*1/*5	17.4	12.3
*5/*5	0	0

Allele frequency	This study	Japanese[8]
*1	91.3	93.8
*5	8.7	6.2

Table 1 shows the performance of our long PCR assay in determining the *CYP2D6*5* genotypes of 46 healthy Japanese subjects, and the distribution of genotypes compared with the previous report in 162 Japanese subjects. This result gave us a similar distribution to the previous report.[8] Direct sequence analyses with the primer walking strategy were carried out to confirm the accuracy of the newly developed method. The sequences obtained from the two samples were in agreement with expected sequences.

The *CYP2D6* gene is extremely polymorphic. To date, more than 100 allelic variants have been described (www.cypalleles.ki.se/cyp2d6.htm). Many of the drugs today are designed to share

more than one drug-metabolizing enzyme, and since many drugs are metabolized by CYP2D6 in clinical studies, there is a need for further study of the *CYP2D6* gene in the future. Most *CYP2D6* mutations except *CYP2D6*5* are SNPs, which are analyzed by simple methods such as TaqMan assay and PCR-RFLP. However, *CYP2D6*5* is based on the deletion of the *CYP2D6* gene, and long PCR assay is required in order to amplify it from the whole gene. Because of the difficulty of amplification, only a few reports about *CYP2D6*5* have been made so far. Population analysis can be efficiently performed through *CYP2D6*5* analysis in addition to the conventional SNP analysis by using the method reported here.

In conclusion, this study developed a long PCR assay for the detection of *CYP2D6*5* without DNA extraction. Since the DNA extraction step was eliminated, the possibility of sample contamination was considerably decreased. Moreover, the saliva sample is not invasive compared to the blood sample. In clinical trials, it will be useful to use non-invasive saliva samples and to discard them as non-medical waste. The genotyping method for *CYP2D6*5* deletion will be useful for a prospective clinical trial when assigning the poor metabolism of *CYP2D6*, and can be utilized as a tool to individualize drug therapy in the near future. An extended population study with *CYP2D6*5* analysis will be necessary to identify the genotype in Japanese individuals.

References

1) Sim, C. S. and Ingelman-Sundberg, M.: The human Cytochrome P450 (CYP) allele nomenclature website: A peerreviewed database of CYP variants and their associated effects. *Hum. Genomics*, **4**: 278–281 (2010).
2) Gervasini, G., Benítez, J. and Carrillo, A. J.: Pharmacogenetic testing and therapeutic drug monitoring are complementary tools for optimal individualization of drug therapy. *Eur. J. Clin. Pharmacol.*, **66**: 755–774 (2010).
3) Teh, K. L. and Bertilsson, L.: Pharmacogenomics of *CYP2D6*: Molecular genetics, Interethnic differences and clinical importance. *Drug Metab. Pharmacokinet.*, **27**: 55–67 (2012).
4) Kiyotani, K., Mushiroda, T., Imamura, C. K., Tanigawara, Y., Hosono, N., Kubo, M., Sasa, M., Nakamura, Y. and Zembutsu, H.: Dose-adjustment study of tamoxifen based on *CYP2D6* genotypes in Japanese breast cancer patients. *Breast Cancer Res. Treat.*, **131**: 137–145 (2012).
5) Okishiro, M., Taguchi, T., Kim, S. J., Shimazu, K., Tamaki, Y. and Noguchi, S.: Genetic polymorphisms of CYP2D6*10 and CYP2C19*2,*3 are not associated with prognosis, endometrial thickness, or bone mineral density in Japanese breast cancer patients treated with adjuvant tamoxifen. *American Cancer Society*, **115**: 952–961 (2009).
6) Karle, J., Bolbrinker, J., Vogl, S., Kreutz, R., Denkert, C., Eucker, J., Wischnewsky, M., Possinger, K. and Regierer, C. A.: Influence of CYP2D6-genotype on tamoxifen efficacy in advanced breast cancer. *Breast Cancer Res. Treat.*, **139**: 553–560 (2013).
7) Kiyotani, K., Shimizu, M., Kumai, T., Kamataki, T., Kobayashi, S. and Yamazaki, H.: Limited effects of frequent *CYP2D6*36-*10* tandem duplication allele on in vivo dextromethorphan metabolism in a Japanese population. *Eur. J. Clin. Pharmacol.*, **66**: 1065–1068 (2010).
8) Kubota, T., Yamaura, Y., Ohkawa, N., Hara, H. and Chiba, K.: Frequencies of *CYP2D6* mutant alleles in a normal Japanese population and metabolic activity of dextromethorphan O-demethylation in different *CYP2D6* genotypes. *Br. J. Clin. Pharmacol.*, **50**: 31–34 (2000).
9) Tateishi, T., Chida, M., Ariyoshi, N., Mizorogi, Y., Kamataki, T. and Kobayashi, S.: Analysis of the *CYP2D6* gene in relation to dextromethorphan O-demethylation capacity in a Japanese population. *Clin. Pharmacol. Ther.*, **65**: 570–575 (1999).
10) Crews, K. R., Gaedigk, A., Dunnenberger, H. M., Klein, T. E., Shen, D. D., Callaghan, J. T., Kharasch, E. D. and Skaar, T. C.: Clinical pharmacogenetics implementation consortium (CPIC) guidelines for codeine therapy in the context of *Cytochrome P450 2D6 (CYP2D6)* genotype. *Clin. Pharmacol. Ther.*, **91**: 321–326 (2012).
11) Tseng, C.-Y., Wang, S.-L., Lai, M.-D., Lai, M.-L. and Huang, J.-d.: Formation of morphine from codeine in Chinese subjects of different *CYP2D6* genotypes. *Clin. Pharmacol. Ther.*, **60**: 177–182 (1996).
12) Steen, V. M., Andreassen, O. A., Daly, A. K., Tefre, T., Børresen, A. L., Idle, J. R. and Gulbrandsen, A. K.: Detection of the poor metabolizer-associated CYP2D6(D) gene deletion allele by long-PCR technology. *Pharmacogenetics*, **5**: 215–223 (1995).
13) Hersberger, M., Jacqueline, M.-J., Rentsch, K. and Hänseler, E.: Rapid detection of the *CYP2D6*3, CYP2D6*4*, and *CYP2D6*6* alleles by tetra-primer PCR and of the *CYP2D6*5* allele by multiplex long PCR. *Clin. Chem.*, **46**: 1072–1077 (2000).
14) Dorado, P., Cáceres, C. M., Pozo-Guisado, E., Wong, M.-L., Licinio, J. and Llerena, A.: Development of a PCR-based strategy for CYP2D6 genotyping including gene multiplication of worldwide potential use. *Biotechniques*, **39**: 571–574 (2005).
15) Hayashida, M., Iwao-Koizumi, K., Murata, S., Yokoyama, A. and Kinoshita, K.: Genotyping of polymorphisms in alcohol and aldehyde dehydrogenase genes by direct application of PCR-RFLP on dried blood without DNA extraction. *Anal. Sci.*, **26**: 503–505 (2010).