Note

Unique Genotyping Protocol of CYP2D6 Allele Frequency Using Real Time Quantitative PCR from Japanese Healthy Women

Madoka Kisoi,[a] Miho Imai,[a] Miwako Yamamura,[a] Yui Sakaguchi,[a] Shigenori Murata,[a,b] Atsushi Ichikawa,[b,c] and Kenji Kinoshita*[,a,b,c]

[a] *School of Pharmaceutical Sciences, Mukogawa Women's University; Kyuban-cho, Koshien, Nishinomiya, Hyogo 663–8179, Japan:* [b] *Institute of Biosciences, Mukogawa Women's University; Kyuban-cho, Koshien, Nishinomiya, Hyogo 663–8179, Japan: and* [c] *Bio Education Laboratory; Higashinakajima, Higashiyodogawa, Osaka 533–0033, Japan.*

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CYP2D6 is an important drug-metabolizing enzyme involved in the metabolism of 20–25% of commonly prescribed drugs. Genetic polymorphism of CYP has clinically significant modifications in patients' drug-metabolizing capacities. Since gene copy number variation (CNV) and single nucleotide polymorphism (SNP) frequently occur in the *CYP2D6* gene, which the activity of CYP2D6 particularly depend on the genetic factors. This study aimed to investigate the frequencies of *CYP2D6* genotypes in a Japanese female subject of 216 healthy volunteers. The volunteers were genotyped for CNV Exon 9 and four *CYP2D6* genetic variants (2*, **5*, **10*, **14*, **41*) performed by TaqMan® genotyping assays. The CNV allele frequencies were 82.9% for two copies, 11.6% for one copy, 4.6% for three copies and 0.9% for zero copy, respectively. The frequencies of *CYP2D6*1*, **2*, **5*, **10*, **14*, and **41* were 38.7, 16.7, 6.3, 34.7, 0.2, and 1.2%, respectively. *CYP2D6*5* and **14* were the major defective alleles. However, this genotyping is labor intensive, time consuming, and costly. We report an optimized novel protocol for the determination of CNV and SNP in *CYP2D6* gene by real-time quantitative PCR. This can lower the cost and accurately determine CNV and SNP in the *CYP2D6* gene with a higher output and enabling reliable estimates of disease prediction in large epidemiological samples.**

Key words CYP2D6; pharmacogenomics (PGx); copy number variation (CNV); single nucleotide polymorphism (SNP); real time quantitative PCR

INTRODUCTION

Genetic polymorphisms in drug-metabolizing enzymes, drug receptors and drug transporters influence an individual's ability to metabolize and excrete drugs. These pharmacogenetic variations contribute inter-individual variability in drug metabolism and response. Among the drug-metabolizing enzyme genes, the greatest impact is due to polymorphisms in drug-metabolizing CYPs such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5. These drug-metabolizing enzymes belong to the CYP superfamily, and they metabolize more than 90% of the commonly prescribed drugs. Notably, determination of *CYP2D6* genotype is important in clinical settings where phenotype prediction and individualization of drug therapy depends on the accuracy of the phenotypes, which are inferred from genotype information. In fact, CYP2D6 is one of the most examined phase I drug-metabolizing enzymes, which 20 to 25% are used clinically such as dextromethorphan and tamoxifen.[1)] For many *CYP2D6* substrate drugs, screening an individual *CYP2D6* genetic makeup and tailoring patient drug therapy can decrease the adverse events while increasing clinical and economic benefits.[2)]

Among the activities of drug-metabolizing enzymes, CYP2D6 is especially affected by genetic factors leading to large inter-individual variability of *CYP2D6* gene polymorphisms produced from over 100 allelic variants.[3–6)]*CYP2D6* is highly polymorphic and variants include single nucleotide polymorphism (SNP), as well as copy number variation (CNV) resulting from *CYP2D6* gene deletion or multiplication. It is said that allele frequencies of *CYP2D6* vary between ethnic population.[7)] The genetic variations in *CYP2D6* result in four different drug metabolism phenotypes; poor metabolizer (PM), intermediate metabolizer (IM), extensive metabolizer (EM), and ultra-rapid metabolizer (UM). Among the allelic variants, there are fully functional alleles (**1* and **2*: rs16947), alleles with reduced function (**10*: rs1065852 and **41*: rs28371725) and null (non-functional) alleles (**5* and **14*: rs5030865). The reported frequencies of *CYP2D6*5* and **14* were less than 10% in the Japanese population.[8)] It causes the PM phenotype and deficient CYP2D6 activity, but duplicates the functional allele leading to increased gene expression and enzyme activity.

Determining the characteristics of *CYP2D6* gene locus, genotypes and phenotypes are extremely important for pharmacogenomics. However, the current CNV and SNP genotyping methods can put constraints on research, since they are inefficient regarding time and cost. Although there are different methods described in the literature for analyzing CNVs,[8–15)] TaqMan® copy number assay is the most common method used to quantify the gene copy number and simplify comparison with other complex assays such as long-range PCR.[16,17)]

The aim of our study was to investigate the frequencies of *CYP2D6* genotypes in a healthy Japanese female population to establish cost-effective unique genotyping protocol for *CYP2D6*. We have examined allele frequencies of the *CYP2D6* CNV (*Exon 9*) as **5* (deletion of the whole *CYP2D6* gene) and its SNPs including **2*, **10*, **14*, and **41* from 216 healthy female subjects.

* To whom correspondence should be addressed. e-mail: kenji_k@mukogawa-u.ac.jp

MATERIALS AND METHODS

Materials Water-soluble paper (120MDP) from Nippon Paper Papylia Co., Ltd. (Tokyo, Japan) and homemade sampling kits "DnaCapture" from Taiko Co., Ltd. (Nishinomiya, Japan), oral care sponge swab from Osaki Medical Co. (Nagoya, Japan) were purchased. Biopsy punch (BPP-40F) from Kai Industries Co., Ltd. (Tokyo, Japan), THUNDERBIRD® probe qPCR Mix (QPK-101) from TOYOBO Co., Ltd. (Osaka, Japan), and TaqMan® Copy Number Assay (assay id: Hs00010001_cn [Ex9]) with an internal control – ribonuclease (RNase) P TaqMan® Copy Number Reference Assay (assay id: 4403326), and TaqMan® Probe and each Primer Mix (**2*: C_27192425_10, **10*: C_11484460_40, **14*: C_30634117D_30, **41*: C__34816116_20) from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.) were used.

Subjects A total of 216 healthy female subjects at Mukogawa Women's University were enrolled in the study. All participants were informed consent in each genotype determination of the allele frequencies of *CYP2D6*1, *2, *5, *10, *14, *41*, and the CNV. The Medical Ethics Committee of Mukogawa Women's University approved the study protocol (No. 17-61).

Sample Preparation Procedure Saliva sampling and subsequent preparation procedures were performed as follows.[18] The subjects were asked to rub their inner cheek surfaces with an oral care sponge swab to collect buccal cells for their genotyping. The swabs including saliva specimens were applied to the homemade sampling kit "DnaCapture" on a part of water-soluble paper and were dried overnight at room temperature. A 4 mmΦ of dried saliva sample was cut with a biopsy punch, directly suspended in 200 μL of distilled water, and heated at 95°C for 5 min. The supernatant of genome DNA template was added to a PCR tube.

The TaqMan®method for *CYP2D6* CNV Genotyping To assess the *CYP2D6* gene copy number, we used a commercial quantitative TaqMan® PCRs with a reaction volume of 10.2 μL were performed once by the QuantStudio™ 12K Flex Real-Time PCR System. The TaqMan® assay mixture consisted of 5 μL of THUNDERBIRD probe qPCR Mix, 0.2 μL of ROX × 50 reference dye, each 0.5 μL of TaqMan® × 20 Copy Number Assay (assay id: Hs00010001_cn [Ex9]) and an internal control – RNase P TaqMan® ×20 Copy Number Reference Assay (assay id: 4403326), and 4 μL of genome DNA template. PCR cycling conditions were as follows: one cycle at 95°C for 10 min, followed by 50 cycles of melting at 95°C for 15 s, and annealed and extended at 60°C for 60 s. Relative quantification was performed using CopyCaller™ Software, following the comparative ΔΔ*Ct* method.

The TaqMan®method for *CYP2D6* SNP Genotyping Genotyping for *CYP2D6*2*, **10*, **14*, and **41* mutant alleles was carried out by allelic discrimination with TaqMan® assays (Table 1). TaqMan® PCRs with a reaction volume of 9.7 μL were performed once by the QuantStudio™ 12K Flex Real-Time PCR System. The TaqMan® assay mixture consisted of 5 μL of THUNDERBIRD probe qPCR Mix, 0.2 μL of ROX × 50 reference dye, 0.5 μL of TaqMan® × 20 probe and each Primer Mix (**2*: C_27192425_10, **10*: C_11484460_40, **14*: C_30634117D_30, **41*: C__34816116_20), and 4 μL of genome DNA template. PCR cycling conditions were as follows: one cycle at 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s, and annealed and extended at 60°C for 60 s. The results were analyzed by the QuantStudio™ 12K Flex Real-Time PCR System TaqMan® Genotyper™ Software.

RESULTS AND DISCUSSION

In this study we describe a simple and reliable allele quantification-based TaqMan® PCR genotyping method that facilitates *CYP2D6* CNV genotyping. Since the SNP genotyping and allele quantification as CNV were performed in this protocol, the PCR assays for SNPs (*CYP2D6*2*, **10*, **14*, **41*) and *CYP2D6* CNV *Exon 9* were optimized to be robust, efficient and reproducible. The allele frequencies of *CYP2D6* CNV and SNPs genotyping were estimated in 216 Japanese young female subjects.

The CNV noted with these samples set were diverse, showing CNV from zero to three genes, respectively. The most frequent CNV allele found in the present study were two copies, followed by one, three and zero copy. In the first attempt of CNV genotyping, a specimen determined to be greater than 1.6 copies and less than 2.4 copies were caluculated by the CopyCaller™ software was defined as 2 copies. Except for two copies of the CYP2D6 gene in the first attempt, total of 47 specimens of CNV assays were to be repeated for triplication as a quality control measure. The result of second achievement on 47 subjects was shown in Fig. 1. These allele frequencies of *CYP2D6* CNV for all 216 subjects were 2 subjects (0.9%) for zero copy, 24 subjects (11.1%) for one copy, 180 subjects (83.3%) for two copies, and 10 subjects (4.6%) for three copies, respectively. Our results also indicate that all these assays should also be useful in detecting the *CYP2D6*5* (gene deletion) in samples that have only one copy due to a whole gene deletion in *CYP2D6*5*.

These frequencies of *CYP2D6*1*, **2*, **5*, **10*, **14*, and **41* were 38.9%, 17.1, 6.3, 36.3, 0.2, and 1.2% in our Japanese female subjects, respectively. The frequency of mutant allele *CYP2D6*14* in our Japanese subjects were very low. Moreover, these results showed that *CYP2D6*10* was the most frequent mutant allele of *CYP2D6*. The observed frequency of *CYP2D6*10* was similar to those of earlier studies,[8,12,19–21] while it was much higher than that in Caucasians.[22] In addition, *CYP2D6*10* and **41*, mainly responsible for the intermediate metabolizers, may play a more important role for the treatment among Japanese patients. In Asians, the most abundant variant allele is *CYP2D6*10* and **41*[19,22–24] which is generally considered to be an allele associated with the IM phenotype. The 19 different genotypes of the CYP2D6 gene present in our subjects were listed together with their respective frequencies in Table 1. *CYP2D6*1/*10* (30.6%), *CYP2D6*1/*1* (16.2%), *CYP2D6*10/*10* (11.6%), and *CYP2D6*1/*2* (9.7%) were the most prevalent genotypes. All the genotypes were in Hardy–Weinberg Equilibrium.

These results suggested that dried saliva samples including genomic DNA had sufficient purity and concentration using TaqMan® Copy Number Assays. The average *Ct* value of *RNase P* of dried saliva samples was 29.8 cycles ±0.8 (data not shown). The genomic DNA copy number in the reaction was calculated from 10^4 to 10^5 copies by a calibration curve (data not shown). Therefore, the genomic DNA templates in saliva samples were good enough to determine CNV. Moreover, the standard deviation of the *Ct* value was in a narrow

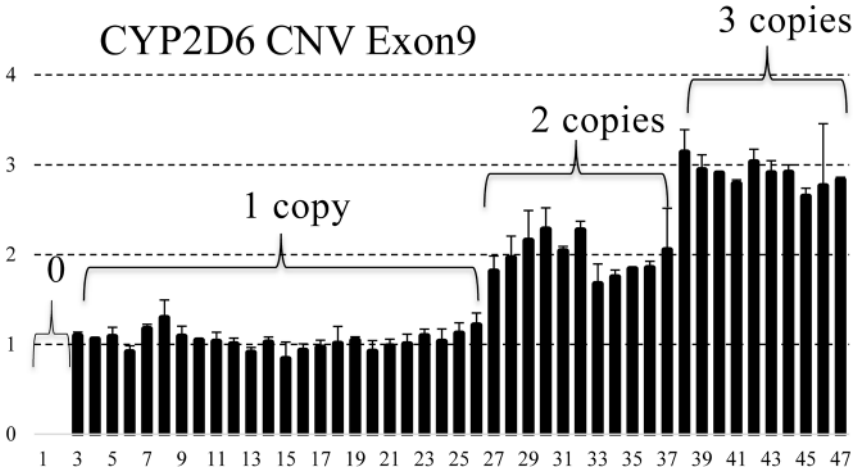

Fig. 1. Comparison of Estimated *CYP2D6* Gene Copy Numbers Using CopyCaller™ Software

Except for two copies (169 subjects within 1.6 to 2.4 copies) of the CYP2D6 gene in the first attempt for all subjects, totally 47 subjects of the CNV assays were repeated for triplication ($n=3$) as a quality control measure. The error bars showed each standard deviation ($n=3$). In terms of final results, these allele frequencies of *CYP2D6* CNV for all 216 subjects were 2 subjects (0.9%) for zero copy, 24 subjects (11.1%) for one copy, 180 subjects (83.3%) for two copies, and 10 subjects (4.6%) for three copies, respectively.

Table 1. Distribution of *CYP2D6* Genotypes in 216 Japanese Subjects

Genotype	Copy No.	*n*	%
**1/*1*	2	35	16.2
*1/*1×2*	3	1	0.5
**1/*2*	2	21	9.7
**1/*5*	1	6	2.8
**1/*10*	2	66	30.6
**1/*10×2*	3	3	1.4
**2/*2*	2	10	4.6
**2/*2×2*	3	1	0.5
**2/*5*	1	5	2.3
**2/*10*	2	17	7.9
**2×2/*10*	3	1	0.5
**2/*10×2*	3	2	0.9
**2/*14*	2	1	0.5
**2/*41*	2	4	1.9
**5/*5*	0	2	0.9
**5/*10*	1	13	6.0
**10/*10*	2	25	11.6
**10×2/*10*	3	2	0.9
**10/*41*	2	1	0.5

range, which means that the amount of genomic DNA introduced into the PCR system was considerably stable according to our sampling procedure using DnaCapture. Also, this new protocol using the homemade sampling kit DnaCapture was lower in cost and has less processing time compared to the general genotyping process. All procedures related to the measurement of CNV *Exon 9* and SNPs (*CYP2D6*2, *10, *14, *41*) of 216 dried saliva samples were completed within two weeks. This sampling kit allowed efficient sample collection and preservation for DNA amplification. It also simplified the sampling process, allowing easier self-sampling.[18] The dried saliva samples can be stored at room temperature for more than one year and their genotyping still could be verified.

We have developed an optimized *CYP2D6* CNV and SNPs genotyping protocol using the water-soluble paper for the real-time quantitative PCR system excluding genomic DNA purification process. The results of this study have demonstrated the capability of using saliva including white blood and buccal cells directly to perform real-time quantitative PCR-based genotyping. The method described here are straightforward, easy to perform, and cost-effective with reduction of time. The total cost of *CYP2D6* gene genotyping for each specimen was less than 10 USD using the homemade sampling kits DnaCapture and commercially available materials. However, it does not include facilities costs depreciation expenses. Most importantly, it overcomes the limitations of previous methods by identifying the duplicated *CYP2D6* allele in heterozygous states with faster prediction.

In conclusion, we constructed a novel protocol to determine the genetic polymorphisms using real-time quantitative PCR. The genotyping of the *CYP2D6* were rapid and suitable for typing both small and large numbers of specimens. The pharmacogenomics approach can be useful in drug development when targeting subgroup patients, who are genetically predisposed to high drug efficacy. Knowing the alleles of the *CYP2D6* in healthy women are clearly important for the future of personalized medicine among breast cancer treatment drugs such as tamoxifen.[21,25–29] For this purpose, the involvement of simple and inexpensive measurement technology is essential.

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Conflict of Interest The authors declare no conflict of interest.

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