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the wax-mediated host-start polymerase chain reaction(PCR)法はCYP2C9*1及び*3アレルの遺伝子型同定を改善する(A Wax-Mediated Hot-Start Polymerase Chain Reaction Improves the Genotyping of CYP2C9* 1 and * 3 Alleles)

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SHORT COMMUNICATION

A Wax-Mediated Hot-Start Polymerase Chain Reaction Improves the Genotyping of *CYP2C9*1* and **3* Alleles

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Cytochrome P4502C9 (*CYP2C9*) is a polymorphic enzyme which metabolizes a variety of medications. The *CYP2C* gene families are extensively homologous ; in particular, the nucleotide sequences in the areas adjacent to polymorphic sites are >95% identical. Frequently, primers designed for amplification of *CYP2C9* also amplify other *CYP2C* genes. Additional amplification of the other *CYP2C* alleles will thus yield an erroneous restriction pattern, resulting in misleading genotype of *CYP2C9*. Thus, a procedure to specifically amplify *CYP2C9* alleles is necessary. In this study, a wax-mediated hot-start polymerase chain reaction (PCR) for the amplification of *CYP2C9*1* and **3* alleles was tested. When serially diluted template DNA was amplified by the hot-start PCR followed by the agarose electrophoresis without the enzyme digestion, the 165 bp band derived from *CYP2C9* sequence was clearly detected without any non-specifically amplified band even from 10 ng DNA template, and the density of this band increased in proportion to the amount of template. On the other hand, when the PCR without the use of wax was performed, the amplification of the allele was irregular. This hot-start PCR gave a significant increase in the sensitivity for the detection of *CYP2C9*1* and **3* alleles. The performance of the present method was studied with 64 Japanese volunteers. After the enzyme digestion of the products obtained by the wax-mediated hot-start PCR, only specific bands corresponding to *CYP2C9*1* and **3* alleles were obtained. This PCR method gave the consistent performance and low background. Thus, the wax-mediated hot-start PCR method should be useful in the genotyping of *CYP2C9*1* and **3*, possibly including *CYP2C9*2* allele in the clinical laboratory.

Key words : *CYP2C9* , genotyping, PCR, wax-mediated

Introduction

Genetic polymorphisms are known to contribute to individual variations of the metabolism of numerous agents[1]. Among the human cytochrome P450 (*CYP*) isoforms, *CYP2C9* is responsible for the metabolism of a number of therapeutic drugs, such as S-warfarin[2], tolbutamide[3] and non-steroidal anti-inflammatory drugs, and detoxificates a carcinogen, benzo[α]pyrene[4,5]. Multiple single-base pair substitution polymorphisms have been discovered in *CYP2C9* cDNA, designated as *CYP2C9*1* (wild type), **2*, **3*, **4*, **5* and **6*[6]. *CYP2C9*1* and **3* alleles appear in Oriental individuals, and *CYP2C9*4* has been recently identified in a Japanese individual[7]. *CYP2C9*3* has an exchange of (A_{1075}→ C) in exon 7 causing an Ile^{359}/Leu amino acid exchange[8]. Cells expressing the *CYP2C9*3* allele have demonstrated significant reductions in maximal elimination rate and increases in Michaeal-Menten constant for many drugs as substrates compared with the wild-type[1,8,9]. Thus, for example, one of the major clinical interests in the polymorphism in *CYP2C9* gene is the association of the metabolism of the anticoagulant drug warfarin with interindividual variability in its therapeutic effect.

After Wang et al.[10] presented a protocol for analysis of a number of mutations based on the polymerase chain reaction (PCR)-endonuclease digestion in the *CYP2C9* gene, several similar methods have been proposed[11,12],

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where a mismatched primer has been designed to detect codon A_{1075}/C (Ile^{359}/Leu) in the PCR. The sequences of *CYP2C* subfamilies, such as *CYP2C9*, *CYP2C18* and *CYP2C19* genes on the chromosome 10[1], are highly homologous. Especially in the areas adjacent to polymorphic sites, the nucleotide sequences are >95% identical[1]. Thus, primers designed for amplification of *CYP2C9* also possibly amplify other *CYP2C* subfamily genes[13]. This additional amplification will thus yield an erroneous restriction pattern, resulting in misleading genotype of *CYP2C9*. Thus, the procedure to specifically amplify *CYP2C9* alleles is essential, especially in view of the potential clinical significance of allelic variants of *CYP2C9* genotype. A wax-mediated hot-start PCR method has been developed to avoid non-specific extension products[14,15]. The wax barrier uses a layer of solid wax to separate the retained reagents and the test sample from the bulk of the reagents until the first heating step of automated thermal cycling melts the wax and consecutively mixes the two aqueous layers. In this study, we examined the wax-mediated hot-start PCR method to specifically amplify *CYP2C9*1* and **3* alleles.

Materials and Methods

1. Reagents and isolation of DNA

Restriction enzymes, *Nsi* I and *Kpn* I, were purchased from New England Biolabs (Beverly, MA, USA). Taq polymerase, 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl and 15 mM $MgCl_2$, pH 8.3) and 2.5 mM of dNTP mix were obtained from Takara (Shiga, Japan). Ampli WAX PCR Gem 100 and 25-bp ladder-marker were purchase from Perkin Elmer (Branchburg, NJ, USA) and Gibco BRL (Rockville, MD, USA), respectively.

The Local Ethics Committee of Asahikawa Medical College has approved this study. Blood samples were drawn from 64 healthy volunteers. Informed consent was obtained from all subjects. Template DNA was isolated from whole blood using a Qiagen maxi kit (Hilden, Germany).

2. PCR endonuclease analysis of *CYP2C9*1* and **3*

For the detection of *CYP2C9*3* allele, the PCR was performed using a *CYP2C9* exon 7 forward primer with 1 bp mismatch (underline) (AATAATAATATGCACGAGGTCCAGAGGTAC) and *CYP2C9* intron 7 reverse primer (GATACTATGAATTTGGGACTTC)[12]. For the *CYP2C9*1* analysis, *CYP2C9* gene fragment was also amplified with an exon 7 forward mismatch primer (AATAATAATATGCACGAGGTCCAGAGATGC) and an intron 7 reverse primer as same as in the *CYP2C9*3* detection[12].

The PCR reaction mixture was separated into two layers with Ampli Wax PCR Gem 100, which was used as a seal reagent and key reaction components were separated ; the lower layer (50 μl) consisted of 25 pmol each primer and 200 μM of dNTPs, and the upper layer (50 μl), 1X PCR buffer, 50 ng of genomic DNA and 2.5 U of Taq polymerase. The PCR amplification parameters followed by the hot-start, consisted of an initial denaturation step at 94°C for 5 min, and 35 cycles of denaturation at 94°C for 20 sec, annealing at 53°C for 10 sec and extension at 72°C for 10 sec. The final extension step at 72°C for 5 min was also performed. The PCR product was digested with each of the restriction enzymes (*Nsi* I or *Kpn* I) to detect *CYP2C9*1* or **3*, respectively, and the DNA fragments were separated on 3.6% nusieve agarose gel followed by staining with ethidium bromide. To compare with the hot-start PCR method, the *CYP2C9*1* and **3* alleles were also detected according to the method (without wax-mediated hot-start) previously described by Sullivan-Klose et al.[12] with the same primer and restriction enzyme sets as used in the hot-start method.

DNA sequence analysis was performed on an Applied Biosystems ABIPRISM 310 Genetic Analyzer.

Results

In order to determine the amplification efficiencies of two PCR methods employed, 6 series of serially diluted template DNA from 6 different individuals were used for each PCR method followed by agarose electrophoresis without the enzyme digestion. Figure 1 gives typical results of electrophoresis. By the wax-mediated hot-start PCR method, the 165 bp band derived from *CYP2C9* allele[16] was clearly detected without any non-specifically amplified band even from 10 ng DNA template, and the density of this band increased in proportion to the amount of template in an ethidium bromide-stained 3.6% nusieve agarose gel (Figs. 1-A and C). The sequence of the 165 bp product was confirmed as derived from *CYP2C9* gene by the sequence analysis. On the other hand, when the PCR without the wax-mediated hot-start was performed, the results of the agarose electrophoresis were not constant (Figs. 1-B and D).

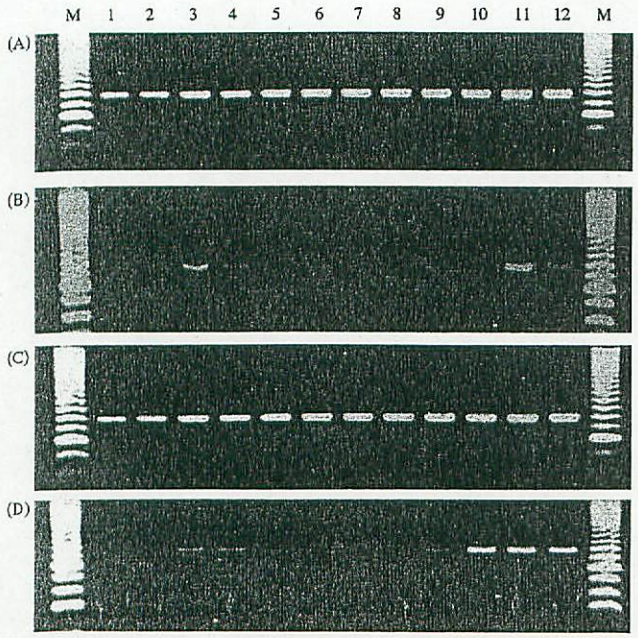

Fig. 1

Electrophoresis of PCR amplified *CYP2C9* fragment (165 bp). PCR amplification was performed by the method with wax-mediated hot-start (A and C) and without the wax-mediated hot-start (B and D). The primer set used was as follows : AATAATAATATGCACGAGGTCCAGAGGTAC with GATACTATGAATTTGGGACTTC (A and B) and AATAATAATATGCACGAGGTCCAGAGATGC with GATACTATGAATTTGGGACTTC (C and D).

Lane M : 25 bp ladder marker, and Lanes 1-12 : 10, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250 and 300 ng (DNA concentration).

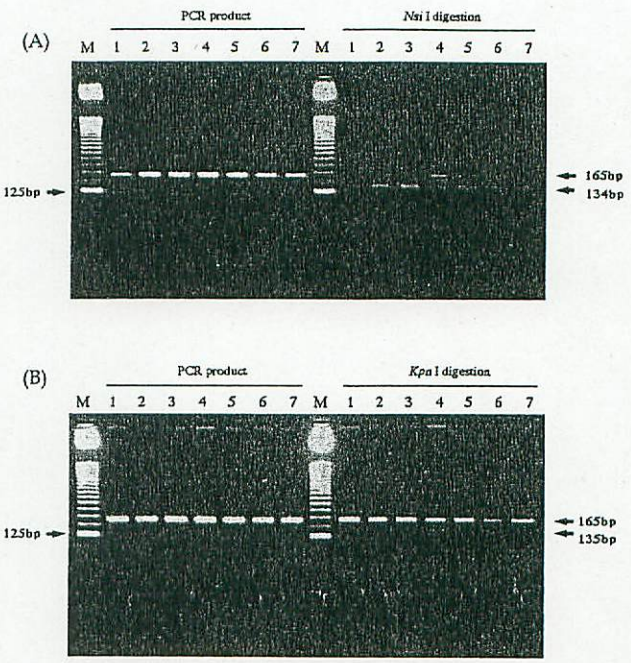

Fig. 2

PCR-RFLP analysis of *CYP2C9*1* and **3*. The PCR product (165 bp) containing A_{1075} allele was digested with *Nsi* I for the detection of *CYP2C9*1* (A), yielded 134 and 31 bp bands. The 165 bp product containing C_{1075} allele was digested with *Kpn* I for the detection of *CYP2C9*3* (B), yielded 135 and 30 bp fragments.

Lane M : 25 bp ladder marker, Lanes 1, 2, 3, 5 and 7 : **1*1* homozygous, and Lanes 4 and 6 : **1*3* heterozygous.

The wax-mediated PCR process yielded a significant increase in the sensitivity of the detection of specific *CYP2C9* alleles.

After the enzyme digestion of the products amplified by the wax-mediated hot-start PCR, specific bands corresponding to *CYP2C9*1* and **3* alleles were obtained as shown in Fig. 2. When 64 Japanese subjects were genotyped, 61 samples showed the homogenous *CYP2C9*1* genotype without any non-specific band, and three samples were heterozygous *CYP2C9*1/*3*.

Discussion

The existence of genetic polymorphisms in *CYP2C9* giving rise to functionally significant effects on enzyme activity is now well established. Impaired metabolism of a low therapeutic index drug, such as S-warfarin, has important clinical consequence. After Wang and co-workers[10] presented a protocol for analysis of a number of mutations based on PCR-endnuclease digestion in the *CYP2C9* gene, several similar methods have been proposed[11,12]. However, extensive sequence homology is evident among *CYP2C* subfamily on the chromosome 10[1]. Especially in the areas adjacent to polymorphic sites, the nucleotide sequences are>95% identical[1]. Thus, primers designed for amplification of *CYP2C9* possibly amplify other *CYP2C* genes[13]. Additional amplification of the other *CYP2C* alleles will thus yield an erroneous restriction pattern, resulting in misleading genotype of *CYP2C9*3*. The instability in the amplification efficiency would be frequently occurred as seen in this study, since the forward primer contained one mismatched nucleotide for the recognition of restriction enzymes (*Nsi* I and *Kpn* I). Thus, the procedure to specifically amplify *CYP2C9* allele is needed.

The hot-start PCR method has been proved to be valuable, especially, for the amplification of longer DNA fragment to avoid the mispriming and the

oligomerization of primers[17]. The use of wax to separate the retained reagent and the test sample from the bulk of the reagent is known to facilitate the hot-start PCR procedure[18]. The present wax-mediated hot-start PCR strikingly increases the specificity and sensitivity of amplifying *CYP2C9* gene fragment. In this study, 61 Japanese individuals were genotyped as homozygous of *CYP2C9*1* and 3 subjects were heterozygous of *CYP2C9*1/*3*. This result was good agreed with the previously reported result in which *CYP2C9*3* allele frequency in the Oriental population has been found to be 0.02[11]. We concluded that the wax-mediated hot-start PCR technique could sensitively and specifically amplify *CYP2C9* gene fragments, possibly including *CYP2C9*2* allele, and that it may be a valuable method in the clinical laboratory. Additionally, the cost of wax was inexpensive.

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