Rapid Single-Nucleotide Polymorphism Detection of Cytochrome P450 (CYP2C9) and Vitamin K Epoxide Reductase (VKORC1) Genes for the Warfarin Dose Adjustment by the SMart-Amplification Process Version 2

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BACKGROUND: Polymorphisms of the CYP2C9 (cytochrome P450 family 2, subfamily C, polypeptide 9) gene (CYP2C9*2, CYP2C9*3) and the VKORC1 (vitamin K epoxide reductase complex, subunit 1) gene (−1639G>A) greatly impact the maintenance dose for the drug warfarin. Prescreening patients for their genotypes before prescribing the drug facilitates a faster individualized determination of the proper maintenance dose, minimizing the risk for adverse reaction and recurrence of thromboembolic episodes. With current methodologies, therapy can be delayed by several hours to 1 day if genotyping is to determine the loading dose. A simpler and more rapid genotyping method is required.

METHODS: We developed a single-nucleotide polymorphism (SNP)-detection assay based on the SMart Amplification Process version 2 (SMAP 2) to analyze CYP2C9*2, CYP2C9*3, and VKORC1 −1639G>A polymorphisms. Blood from consenting participants was used directly in a closed-tube real-time assay without DNA purification to obtain results within 1 h of blood collection.

RESULTS: We analyzed 125 blood samples by both SMAP 2 and PCR-RFLP methods. The results showed perfect concordance.

CONCLUSIONS: The results validate the accuracy of the SMAP 2 for determination of SNPs critical to personalized warfarin therapy. SMAP 2 offers speed, simplicity of sample preparation, the convenience of isothermal amplification, and assay-design flexibility, which are significant advantages over conventional genotyping technologies. In this example and other clinical scenarios in which genetic testing is required for immediate and better-informed therapeutic decisions, SMAP 2-based diagnostics have key advantages.

Warfarin is the most widely prescribed anticoagulant for the treatment of thromboembolic disorders. Because of the narrow therapeutic index and the large individual variation observed between warfarin dose and its anticoagulant effect (1), a careful adjustment of the dose on the basis of the prothrombin time expressed as the international normalized ratio (PT-INR) is essential. Subgroup analyses in a number of studies have shown that the risk of bleeding increases sharply when the PT-INR is greater than the upper limit of the therapeutic interval (2–4), and the risk of thromboembolic events increases when the PT-INR falls below the therapeutic interval (4, 5). To prevent adverse events requires immediate adjustment of the warfarin dosage. Establishing a
proper maintenance dose is challenging, however, because of widespread interindividual variation in the response to warfarin. This variation is explained in part by the genotype for the target site of warfarin action (pharmacodynamic effects) and by the genotype for the individual’s metabolizing enzymes (pharmacokinetic effects).

Subunit 1 of the vitamin K epoxide reductase complex (VKORC1), a component of vitamin K epoxide reductase (VKOR), is a chief molecular target of warfarin (6). VKOR reportedly is a multisubunit enzyme, but a single peptide, VKORC1, may be responsible for its reductase activity (7). This enzyme recycles vitamin K 2,3-epoxide to vitamin K hydroquinone, which is required by γ-glutamyl carboxylase for the posttranslational modification of blood coagulation factors II, VII, IX, X, and others. Recent findings have shown that polymorphisms in VKORC111 (vitamin K epoxide reductase complex, subunit 1) have a large impact on warfarin dose (8–13). In our previous study (14), we found that 3 VKORC1 polymorphisms (−1639G>A, 1173C>T, and 1542G>C) were invariantly linked. The haplotypes can be categorized into M1, M2, and M3 groups, as Rieder et al. previously observed (13). Individuals in the M3 group, which has the −1639AA genotype, require a lower maintenance dose of warfarin than individuals in the other groups. A multivariate analysis clearly showed that this polymorphism was the most important determinant of daily warfarin dose, explaining 16.5% of the variation. From these findings, we concluded that genotyping can be simplified considerably through the use of the −1639G>A polymorphism as the chief pharmacodynamic marker for predicting warfarin sensitivity.

Clinically available warfarin is a racemic mixture of S- and R-warfarin, and the potency of S-warfarin is 3- to 5-fold higher than that of R-warfarin (1, 15). S-warfarin is metabolized to 7-hydroxywarfarin predominantly by the polymorphic enzyme encoded by the CYP2C9 gene (cytochrome P450, family 2, subfamily C, polypeptide 9), whereas R-warfarin is metabolized by multiple cytochrome P450s, including CYP1A2 and CYP3A4 (16, 17). The enzymatic activity of CYP2C9 has a substantial influence on the observed anticoagulant effect of S-warfarin, the primary active form of the drug. Previous findings revealed that the common functional variants encoded by single-nucleotide polymorphisms (SNPs) CYP2C9*2 (430C>T, exon 3) and CYP2C9*3 (1075A>C, exon 7) have approximately 70% and 10%, respectively, of the metabolic capacity of the enzyme encoded by the wild-type (Wt) gene (CYP2C9*1) (18, 19).

In 2007, the US Food and Drug Administration updated the labeling recommendations for warfarin to stress that genetic information is helpful for improving the initial estimate of warfarin dose for individual patients (20), after several randomized prospective clinical trials showed the benefit of genotype-guided warfarin prescription (21, 22).

Warfarin therapy typically commences soon after diagnosis of a disease for which this drug has a clinical indication; therefore, testing with a short turnaround time, ideally within 1 h of diagnosis, should be made available. Numerous strategies have been developed for SNP discrimination (Table 1) (23–32), but no method has eliminated background amplification or DNA purification so that CYP2C9 and VKORC1 genotypes can be diagnosed within 1 h of blood collection. Recently, we reported the Smart-Amplification Process version 2 (SMAP 2), which can detect SNPs after about 30 min of incubation under isothermal conditions (33). We adapted

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*NASBA, nucleic acid sequence–based amplification; LCR, ligase chain reaction; SDA, strand-displacement amplification; RCA, rolling-circle amplification; bDNA, branched DNA; O, .

11 Human genes: VKORC1, vitamin K epoxide reductase complex, subunit 1; CYP2C9, cytochrome P450, family 2, subfamily C, polypeptide 9; CYP2C8, cytochrome P450, family 2, subfamily C, polypeptide 8; CYP2C19, cytochrome P450, family 2, subfamily C, polypeptide 19.
the SMAP 2 by designing primer sets to detect the VKORC1 \(-1639G>A\), CYP2C9*2, and CYP2C9*3 genetic polymorphisms.

Materials and Methods

CLINICAL SAMPLES
The DNA-analysis study was approved by the Institutional Review Board for clinical trials at Gunma University Hospital and the Ethical Committee for Human Genome Analysis at Gunma University. Written consent was obtained from all participants after they had been informed of the experimental procedure and the purpose of the study. Two milliliters of blood was drawn from healthy Japanese volunteers and unrelated Japanese patients treated with warfarin at the Department of Cardiovascular Medicine, Gunma University Hospital, Maebashi, Japan, and was anticoagulated with 4 mg dipotassium EDTA. Blood samples were analyzed immediately or stored at \(-80^\circ\text{C}\) until assayed.

Blood samples were also obtained from Swedish patients being treated at the anticoagulation clinic at Uppsala University Hospital and from the Swedish warfarin genetics (WARG) cohort at Karolinska Institute. Approval was obtained from the appropriate ethics committees. These blood samples were anticoagulated and stored as described above.

DETECTION OF VKORC1 \(-1639G>A\), CYP2C9*2, AND CYP2C9*3 POLYMORPHISMS BY THE SMAP 2
The SMAP 2 is a unique genotyping technology that can detect a germline genetic mutation in a single step after about 30 min of incubation under isothermal conditions \((34)\). A set of specifically designed primers, designated the turn-back primer, the folding primer, the boost primer, 2 outer primers, and the competitive probe \((34)\), enables allele-specific amplification. The turn-back primer contains a sequence at the 3’ end complementary to the target sequence and another sequence at the 5’ end that is complementary to a sequence present on the same DNA strand but situated approximately 15–40 bp further downstream. The folding primer consists of a sequence at the 3’ end complementary to a target genomic sequence and a sequence at the 5’ end that self-anneals to create a hairpin structure. Outer primers strand-displace the DNA synthesized from the turn-back primer and the folding primer. These synthesized DNAs lead to a self-primed DNA synthesis. The role of the boost primer is to accelerate the speed of amplification. For suppression of background amplification, the SMAP 2 uses 2 strategies: the use of an asymmetrical primer design, which minimizes misamplification pathways, and inclusion of Taq MutS in the reaction mixture. Taq MutS is a mismatch-binding protein that recognizes mismatched duplexes between the target DNA and any primer, such as an SNP-discrimination primer (turn-back, folding, or boost primer). Binding of this protein to misprimed templates creates a barrier to the strand-displacing DNA polymerase, preventing exponential amplification of those molecules, thereby reducing background.

The SMAP 2 assay was carried out in a 25-\(\mu\text{L}\) reaction mixture containing 3.2 \(\mu\text{mol/L}\) folding primer, 3.2 \(\mu\text{mol/L}\) turn-back primer, 1.6 \(\mu\text{mol/L}\) boost primer, 0.4 \(\mu\text{mol/L}\) of each outer primer, 16 \(\mu\text{mol/L}\) competitive probe (in the case of CYP2C9*2), 1.4 \(\mu\text{mol/L}\) of each deoxynucleoside triphosphate (dNTP), 50 mL/L DMSO, 20 mmol/L Tris-HCl (pH 8.0), 10 mmol/L KCl, 10 mmol/L \((\text{NH}_4)_2\text{SO}_4\), 8 mmol/L MgSO\(_4\), 1 mL/L Tween® 20, SYBR® Green I (Molecular Probes) diluted to 1 part in 100 000, 6 U Taq DNA polymerase (K.K. DNAFORM), and 0.3 \(\mu\text{g}\) MutS (K.K. DNAFORM). The templates used for the SMAP 2 assays were prepared by mixing 1 volume of whole blood with 2 volumes of 150 mmol/L NaOH, vortex-mixing, and incubating at 98 °C for 5 min. The sample preparation was chilled on ice, and 0.5 \(\mu\text{L}\) was added directly into a reaction mixture. SMAP 2 reactions were assembled on ice and incubated at 60 °C for 60 min. The Mx3000P system (Stratagene) or the ABI 7500 Fast Real-Time PCR instrument (Applied Biosystems) was used to maintain isothermal conditions and to monitor the change in fluorescence intensity due to intercalation of the SYBR Green I dye during the reaction.

DETECTION OF VKORC1 \(-1639G>A\), CYP2C9*2, AND CYP2C9*3 POLYMORPHISMS BY PCR-RFLP
Genomic DNA was extracted from whole blood with a QiAamp Blood Kit (Qiagen). For the VKORC1 \(-1639G>A\) polymorphism, PCR was carried out in a 25-\(\mu\text{L}\) volume containing 0.1 \(\mu\text{g}\) genomic DNA, 12.5 pmol of each primer (\(5’\)-ATCCCTCTGGGAAGT-CAAGC-3’ and \(5’\)-CACCTTCAACCTCCTCCATCC-3’; Kurabo), 0.2 nmol/L of each dNTP, PCR Gold Buffer (Applied Biosystems), 37.5 nmol/L MgCl\(_2\), and 1 U Taq DNA polymerase (Applied Biosystems). The cycling profile used for all reactions consisted of an initial step at 95 °C for 5 min; 35 cycles of 95 °C for 60 s, 60 °C for 30 s, and 72 °C for 2 min; and a 10-min final extension at 72 °C. The resulting 636-bp product was digested for 1 h with 20 U NciI at 37 °C and analyzed by electrophoresis in a 20-\(\mu\text{L}\) agarose gel. NciI cut PCR products containing the \(-1639A\) allele into 522- and 114-bp fragments and cut PCR products containing the \(-1639G\) allele into 472-, 50-, and 114-bp fragments.

For the CYP2C9*2 polymorphism, PCR was carried out in a 25-\(\mu\text{L}\) volume containing 0.1 \(\mu\text{g}\) genomic...
DNA, 10 pmol of each primer (5′-GGTATGGTGGCAATGAGAATCTCGTGCACAC-3′ and 5′-GGGTTGTTTCTCACTGCTAACTGATT-3′; Kurabo), 5.0 nmol/L of each dNTP, PCR Gold Buffer (Applied Biosystems), 37.5 mmol/L MgCl₂, and 1U Taq DNA polymerase (Applied Biosystems). The cycling profile consisted of an initial step at 94 °C for 9 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a 5-min final extension at 72 °C. The resulting 454-bp product was digested for 1 h with 20 U AvaII at 37 °C and analyzed by electrophoresis in a 20-g/L agarose gel. AvaII cut the PCR products containing the 430C allele into 397- and 57-bp fragments and did not cut PCR products containing the 430T allele.

For the CYP2C9*3 polymorphism, the PCR was the same as that for CYP2C9*2, but the primer sequences were 5′-TGCAACCGTGCCAGGTTACG-3′ and 5′-ACAAATCTACCTTGGGAAATGAG-3′ (Kurabo). The cycling profile consisted of an initial step at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and a 7-min final extension at 72 °C. The resulting 454-bp product was digested for 1 h with 20 U KpnI at 37 °C and analyzed by electrophoresis on a 20-g/L agarose gel. KpnI did not cut PCR products containing the 430C allele into 397- and 57-bp fragments and did not cut PCR products containing the 430T allele.

Results

IDENTIFICATION OF THE VKORC1 −1639G>A POLYMORPHISM BY SMAP 2

Fig. 1 shows the locations and sequences of the primers for SMAP 2–based detection of the VKORC1 −1639G>A SNP. The discrimination primers used to recognize the Wt and mutant (Mt) alleles of the VKORC1 polymorphism were of different designs. The Wt allele (−1639G) was recognized by the 5′ end of the turn-back primer (TP) W and folding primer (FP) M were designed to be Wt allele–specific and Mt allele–specific, respectively. Arrows indicate the nucleotides corresponding to the SNP site. The FP has a specific sequence at the 5′ end (lowercase letters) to enable self-annealing hairpin formation. Only 1 outer primer (OP) is used in the Mt allele assay. BP, boost primer.

DNA, 10 pmol of each primer (5′-GTATTCTTGCGCTGAAACCCATA-3′ and 5′-GGCTTGGTTCTCAACTC-3′; Kurabo), 5.0 nmol/L of each dNTP, PCR Gold Buffer (Applied Biosystems), 37.5 mmol/L MgCl₂, and 1U Taq DNA polymerase (Applied Biosystems). The cycling profile consisted of an initial step at 94 °C for 9 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a 5-min final extension at 72 °C. The resulting 454-bp product was digested for 1 h with 20 U AvaII at 37 °C and analyzed by electrophoresis in a 20-g/L agarose gel. AvaII cut the PCR products containing the 430C allele into 397- and 57-bp fragments and did not cut PCR products containing the 430T allele.

For the CYP2C9*3 polymorphism, the PCR was the same as that for CYP2C9*2, but the primer sequences were 5′-TGCAACCGTGCCAGGTTACG-3′ and 5′-ACAAATCTACCTTGGGAAATGAG-3′ (Kurabo). The cycling profile consisted of an initial step at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and a 7-min final extension at 72 °C. The resulting 105-bp product was digested for 1 h with 20 U KpnI at 37 °C and analyzed by electrophoresis on a 20-g/L agarose gel. KpnI did not cut PCR products containing the 1075A allele but did cut PCR products containing the 1075C allele into 85- and 20-bp fragments.

Fig. 1. VKORC1 sequence and the primer set for VKORC1 typing.

(A), Sequence of the VKORC1 gene. Arrow indicates position of the SNP nucleotides of the VKORC1 allele (−1639G→A). Heavy lines indicate DNA sequences used for primer design. (B), Primer set for SNP typing of the VKORC1 allele. Turn-back primer (TP) W and folding primer (FP) M were designed to be Wt allele–specific and Mt allele–specific, respectively. Arrows indicate the nucleotides corresponding to the SNP site. The FP has a specific sequence at the 5′ end (lowercase letters) to enable self-annealing hairpin formation. Only 1 outer primer (OP) is used in the Mt allele assay. BP, boost primer.
dent after 40 min (Fig. 2) and was reproducible. Conversely, similar amplification kinetics were observed for the VKORC1 –1639A (Mt) SMAP 2 assay. Conclusive results from homozygous Mt and heterozygous (Wt/Mt) blood samples were obtained after approximately 30 min, and the results reached a plateau at approximately 40 min. Misamplification from homozygous Wt blood samples did not become apparent even after 40 min of incubation, however. Hence, SNP calls for the VKORC1 SMAP 2 assays were based on a standard detection time of 40 min.

To demonstrate the accuracy and clinical utility of these assays for handling multiple samples, we assayed 125 blood samples by both the SMAP 2 and PCR-RFLP methods. Of the 125 samples, 1 person was homozygous Wt, 25 were heterozygous (Wt/Mt), and 99 were homozygous Mt (Mt/Mt). All of the data for the SMAP 2 and PCR-RFLP analyses showed perfect concordance.

**DISCRIMINATION OF SUBFAMILY GENES AND IDENTIFICATION OF CYP2C9 POLYMORPHISMS BY SMAP 2**

Members of the large gene family encoding the cytochrome P450 proteins are challenging to distinguish from each other by hybridization or amplification techniques. In the case of CYP2C9, it is necessary to discriminate the signals from those for the highly related subtypes CYP2C8 (cytochrome P450, family 2, subfamily C, polypeptide 8), CYP2C18 (cytochrome P450, family 2, subfamily C, polypeptide 18), and CYP2C19 (cytochrome P450, family 2, subfamily C, polypeptide 19), because all 4 genes have a high degree of sequence similarity. We selected SMAP 2 primer sequences that were unique to the CYP2C9 subtype to enable amplification of this subtype alone; these primer sequences imperfectly matched the other 3 family members (CYP2C8, CYP2C18, and CYP2C19). The locations and sequences of the primers for CYP2C9*2 and CYP2C9*3 are shown in Figs. 3 and 4, respectively.

For the CYP2C9*2 and CYP2C9*3 polymorphisms, we engineered the folding primer to be the SNP-discrimination primer, and its 3’ terminal nucleotide recognized both Wt and Mt alleles. We analyzed 125 blood samples. For the CYP2C9*2 polymorphism, 123 were homozygous Wt (Wt/Wt), 1 was heterozygous (Wt/Mt), and 1 was homozygous Mt (Mt/Mt). For the CYP2C9*3 polymor-
phism, 116 were homozygous Wt, 8 were heterozygous (Wt/Mt), and 1 was homozygous Mt. All of the data for the 125 human blood samples were verified by PCR-RFLP analyses, which demonstrated perfect concordance with the SMAP 2 results and confirmed the SMAP 2 assay’s high specificity for the \textit{CYP2C9} alleles.

The amplification time course was similar to that of \textit{VKORC1} assays (Fig. 2), and misamplification was never observed, even after 40-min incubations.

**Discussion**

In this study, we established a SMAP 2–based diagnostic method to detect \textit{VKORC1} \textit{C1639A}, \textit{CYP2C9*2}, and \textit{CYP2C9*3} genetic polymorphisms, which are important for evaluating adjustments in warfarin dosage. Our method was able to detect these 3 SNPs (or Wt sequences) within 1 h with <1 μL of whole blood per assay. The SMAP 2 is a polymerase-based chain reaction that uses a unique primer design and a strand-displacing polymerase for amplification of target DNA sequences under isothermal conditions. In combination with the unique background-suppression capabilities of MutS, the SMAP 2 can detect a particular SNP by DNA amplification alone, with no requirement for any downstream analysis, such as restriction enzyme treatment, electrophoresis, or probe hybridization. Furthermore, commonly used PCR-based techniques usually require careful DNA extraction, because impurities interfere with the enzymatic activity of \textit{Taq} DNA polymerase. The SMAP 2, however, uses the enzyme \textit{Aac} polymerase, which is highly resistant to cellular contaminants; hence, the assay works directly on blood samples, which require only a simple initial heat-denaturation step. Furthermore, the specificity of the SMAP 2 is dictated by the amplification process: Detection of amplified DNA with an intercalating fluorescent dye is sufficient for making an SNP determination. Blood sample preparation and assay setup generally take 20 min, and the SMAP 2 reaction itself takes 40 min. Reaction times vary slightly for different genes, depending on primer melting temperatures or the conformations of the primers themselves. These are the common factors in the setup of the SMAP 2 assay.
that affect the amplification efficiency in primer-based amplification methods. The SMAP 2 demonstrated several advantages over conventional genotyping technologies for genotyping warfarin dose–related genes. One of the strong points is the flexibility of primer design. Loop-mediated isothermal amplification (LAMP) is a rapid SNP-typing method similar to ours; however, this method requires the use of 2 SNP-discrimination primers that must hybridize around the SNP. In CYP2C9 genotyping, for example, the DNA sequence around the SNP is the same as in the CYP2C19 gene (Fig. 3). Consequently, the LAMP assay’s inner primers cannot hybridize to CYP2C9 selectively because the 2 genes have identical sequences in this critical area. Because the SMAP 2 requires only a single discrimination primer for genotyping SNPs, it has greater design flexibility and is better suited for subfamily discrimination. In our method, we engineered the folding primer to be the SNP-discrimination primer and placed the turn-back primer 50 bp downstream from the SNP site to discriminate CYP2C9 from other members of the subfamily. Our SNP-detection primer (i.e., the folding primer) is by itself not specific for CYP2C9 for the same reason as the LAMP primer, but because it is used in combination with the turn-back primer, the assay is specific for CYP2C9.

It is important to note that CYP2C9 metabolizes not only S-warfarin but also many other drugs, including phenytoin (35), tolbutamide (36), losartan (37), and nonsteroidal antiinflammatory drugs (38–40). Hence, the SMAP 2–based CYP2C9 assay used in this study may also be useful for similar pharmacokinetics studies of the clinical use of these and other drugs.

The methods we have described make possible rapid and accurate genotyping in the clinical setting as well as adjustments of warfarin dose based on genetic information. For more personalized medical care and improvement in the efficacy and safety of anticoagulation therapy, SMAP 2–based diagnostics may become an important technology for future point-of-care testing.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Fig. 4. The primer set for CYP2C9*3 typing and sequence alignment of CYP2C9 subfamily genes.

(A), Sequence alignment of CYP2C9 subfamily genes. Dots show positions for which the nucleotides are identical to those in the CYP2C9 sequence. Arrow indicates position of SNP nucleotides (1075A>H11022C). Heavy lines on the sequences indicate primer regions. (B), Primer set for SNP typing of the CYP2C9*3 allele. Folding primers (FPs) W and M were designed to be specific for the Wt allele and the Mt allele, respectively. The FP has a specific sequence at the 5’ end (lowercase letters) to enable self-annealing hairpin formation. Arrows indicate the nucleotides corresponding to SNP sites.
References


