Polymorphism of Warfarin Related Genes (VKORC1 and CYP2C9) Analyzed by the Smart Amplification Process Method in Patients with Atrial Fibrillation

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Abstract
The use of warfarin has been established in the prevention of thromboembolic events, but the maintenance dose varies among individual patients. The polymorphism of two genes (VKORC1 and CYP2C9) involved in the pharmacodynamics and pharmacokinetics of warfarin is considered to be one reason for the differences in the individual maintenance dose. Although single nucleotide polymorphism (SNP) analysis can be performed using various methods, all are time-consuming and require considerable effort. Recently, a rapid SNP detection system has been developed called the smart amplification process (SmartAmp) method. Two hundred and sixty-three patients receiving anticoagulation therapy were genotyped for VKORC1 (−1639G>A) and CYP2C9*3 (1075A>C) using this SmartAmp method. SNP analysis using the SmartAmp method can be performed in one step and within 1 hour. For the VKORC1 (−1639G>A) allelic variation, the number of patients with the GG, GA, and AA genotypes were 2 (0.8%), 68 (25.8%), and 193 (73.4%), respectively. For the CYP2C9*3 (1075A>C) allelic variation, the number of patients with the AA, AC, and CC genotypes were 257 (97.7%), 6 (2.3%), and 0 (0%), respectively. These results were almost identical to those obtained previously in a Japanese population. The SmartAmp method is reliable, easy to use, and may enable cardiologists to prescribe the appropriate dose of warfarin based on SNP results during the patients’ first clinic visit.

Key Words
warfarin, VKORC1, CYP2C9, SmartAmp, single nucleotide polymorphism

Introduction
Warfarin is the most widely prescribed oral anticoagulation agent for the treatment of thromboembolic disorders. Because warfarin has a narrow therapeutic index and there is large individual variability between its dose and anticoagulant effect, careful dose adjustment is essential to manage patients based on the prothrombin time (PT) expressed as the international normalized ratio (INR). The polymorphism of two genes (VKORC1 and CYP2C9) that are involved in the pharmacodynamics and pharmacokinetics of warfarin is thought to be one reason for the different warfarin maintenance doses required for individuals. Although single nucleotide polymorphism (SNP) analysis has

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been performed using various methods\textsuperscript{14,16}, a lengthy time and laborious efforts are required before the results are known. Two to 3 weeks are generally needed for commercial laboratories to genotype \textit{VKORC1} or \textit{CYP2C9}. Physicians therefore must start warfarin therapy without full information because of the delay in receiving the genetic results. Because an overdose of warfarin can result in hemorrhage, physicians may prescribe a lower than optimal dose to avoid this. If the starting dose is too low, it takes much longer to achieve satisfactory anticoagulation status, and requires frequent patient visits for PT-INR confirmation tests. Moreover, patients with poor anticoagulation status may be at increased risk for the occurrence of thrombotic events. If the necessary genetic information could be obtained during the first day of a clinic visit, the risk for adverse events would be greatly reduced. Under these circumstances, a rapid method for SNP analysis has long been anticipated.

**Patients and Methods**

Two hundred and sixty-three patients regularly treated at the Division of Cardiology of St. Marianna University Hospital and Yokohama City Seibu Hospital were enrolled in this study. All patients with stable anticoagulation on a target INR of between 1.6 and 2.6 were investigated.

All patients were genotyped for \textit{VKORC1} (–1639G>A) and \textit{CYP2C9} (1075A>C) using the rapid SNP detection system called the SmartAmp method\textsuperscript{16}. The SmartAmp method amplifies nucleic acid and detects variations in it and was jointly developed by DNAFORM, Inc. (Yokohama, Japan) and the Institute of Physical and Chemical Research (Yokohama, Japan). \textit{VKORC1} and \textit{CYP2C9} Typing Kit was provided from DNAFORM Inc. Blood samples were collected during routine blood tests for PT-INR. SNPs were directly detected from 5 \( \mu \)L blood samples according to the instructions of the developers. Briefly, 5 \( \mu \)L of blood were added to 10 \( \mu \)L of pretreatment solution, heated at 98°C for 5 min, and then cooled to 4°C. One microliter of the sample was added to each reaction mixture (final volume 50 \( \mu \)L), i.e., primers for \textit{VKORC1} (G), \textit{VKORC1} (A), \textit{CYP2C9} (A), and \textit{CYP2C9} (C), placed in an ABI PRISM 7000 (Applied Biosystems, Tokyo, Japan), and amplified at 60°C for 40 min. SNP results were determined based on the combined amplification results.

This study was approved by the Ethics Committee of St. Marianna University School of Medicine (Approval No. 1299), and written informed consent was obtained from all patients prior to blood sampling.

**Results**

Fig. 1 shows a representative reaction profile for the determination of \textit{VKORC1} and \textit{CYP2C9} genotypes in one patient using the SmartAmp method. A supplemental explanation of SmartAmp method mechanism is given here, since it is necessary to understand this figure.

Primers are target specific, and react to the target site of DNA. The amplification of double-strand DNA is marked by the intercalation of

![Image](https://via.placeholder.com/500)

Fig. 1. Representative reaction profile for the determination of \textit{VKORC1} and \textit{CYP2C9} genotypes using the SmartAmp method. For \textit{VKORC1} genotyping, both products were amplified with the wild-type (G) (line 1) and mutant (A) (line 2) allele-specific primers. The results indicated that the patient was a heterozygous carrier (\textit{VKORC1} [GA]). For \textit{CYP2C9} genotyping, although products amplified with the wild-type (\textit{CYP2C9} 1A) allele-specific primer were observed (line 3), products with the mutant (\textit{CYP2C9} 3C) allele-specific primer were not obtained (line 4), indicating that the patient was a homozygous carrier (\textit{CYP2C9} [AA]). By combining these amplification results, the genotypes of this patient were determined to be \textit{VKORC1} (GA) and \textit{CYP2C9} (AA).
SYBR Green I and monitored by the ABI PRISM 7000 system. Any increase in the amount of reactive products is detected and recorded continuously over time. Four different primers were allowed to react in separate tubes in this study, and their results were combined in the ABI PRISM 7000 system. The final results are shown in a single figure with four different colored lines, as shown in Fig. 1. The vertical axis shows the amount of reactive products, while the horizontal axis shows reaction time.

For VKORC1 genotyping, both products were amplified with the wild-type (G) (line 1) and mutant (A) (line 2) allele-specific primers. The results indicated that the patient was a heterozygous carrier (VKORC1 [GA]). For CYP2C9 genotyping, although products amplified with the wild-type (CYP2C9*1, A) allele-specific primer were observed (line 3), no products with the mutant (CYP2C9*3, C) allele-specific primer were obtained (line 4), indicating that the patient was a homozygous carrier (CYP2C9[AA]).

By combining these amplification results, the genotypes of this patient were determined to be VKORC1 (GA) and CYP2C9 (AA). There are 9 combination patterns indicating the genotyping results. Among the patients in the present study, 257 (97.7%) were homozygous CYP2C9*1 carriers (AA) and 6 (2.3%) were heterozygous carriers of the CYP2C9*1 and CYP2C9*3 alleles (AC). No homozygous CYP2C9*3 carriers (CC) were found as shown in Table 1. For the F’KORC1 (−1679G>A) allelic variation, the number of patients with the GG, GA, and AA genotypes were 2 (0.8%), .68 (25.8 %), and 193 (73.4%), respectively.

### Discussion

DNA purification is not necessary before performing SNP analysis of individual samples using the SmartAmp method. The pretreatment time is only 5 minutes, and the subsequent amplification reaction requires 40 minutes. The SNP results are available after these two simple procedures. Therefore, a diagnosis can be made within 1 hour after blood sampling.

Another advantage of this SmartAmp method is that it requires only a small amount of blood. In addition to the small sample volume, another convenient feature is that whole blood can be used without any laborious pre-treatments. This allows the utilization of residual samples or a few drops of blood from the puncture site.

The simplicity of sample processing is important when the general medical laboratory workload is taken into account. The SmartAmp method is expected to become widespread among healthcare institutions, because it is very easy to learn and to perform routinely.

Our genotyping results were nearly identical to those previously reported from among the Japanese population. Although we did not perform a validation study with our samples, the similarity of the results with previously reported and validated ones indicate the reliability of the SmartAmp method.

Numerous strategies have been developed for SNP determination, but none provided VKORC1 and CYP2C9 genotyping within 1 hour of blood sampling without DNA purification and elimination of the background (nonspecific) amplification. This new technique will enable us to prescribe pa-
tients the appropriate warfarin dose on the day of the first clinic visit based on accurate SNP information. Although the SmartAmp method is not yet commercially available, it is anticipated that it will contribute to making routine hospital laboratory work faster and more convenient. Because early knowledge of genetic information on individual patients will reduce such adverse complications as the risk of thrombotic event occurrence with warfarin underdosing and massive hemorrhage with overdosing, a rapid genotyping method will offer major advantages in patient care.

In conclusion, the SmartAmp method is impressively rapid, easy to use and reliable.

References


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Rapid genotyping of \textit{VKORC1} and \textit{CYP2C9}