Ugotavljanje VKORC1 polimorfizma: Primerjava metod verižne reakcije s polimerazo/ polimorfizma dolžin restrikcijskih fragmentov (PCR + RFLP) z alelno specifično verižno reakcijo s polimerazo

Detection of vkorc1 polymorphism: comparison of polymerase chain reaction/restriction fragment length polymorphism (pcr + rflp) with allele–specific polymerase chain reaction

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Izvleček

Namen: VKORC1 polimorfizem je pomemben genetski dejavnik, ki vpliva na zahtevan odmerek varfarina pri bolnikih, ki potrebujejo različne odmerke varfarina za doseganje antikoagulacijskega terapevtskega učinka. Cilj naše raziskave je bil na vzorcu splošne populacije ugotoviti frekvenco enonukleotidnega polimorfizma (SNP) v VKORC1 genu na preprost, hiter in ekonomičen način.

Metode: Za genotipizacijo smo uporabili metodo verižne reakcije s polimerazo oz. polimorfizem dolžin restrikcijskih fragmentov (PCR/RFLP), ki smo jo primerjali z metodo alelno specifične verižne reakcije s polimerazo. Genotipizirali smo 441 vzorcev genomske DNA preiskovancev iz severovzhodne Slovenije. Ocenili smo, ali je izbrana skupina v Hardy–Weinbergovem ravnovesju, ter izračunali posamezne genotipe in frekvenco alelov.

Abstract

Purpose: The VKORC1 polymorphism is an important genetic factor affecting warfarin dose requirement. Patients require different warfarin doses in order to achieve the target therapeutic anticoagulation. The aim of our study was to determine the frequency of single nucleotide polymorphisms (SNP) in the VKORC1 gene in the general population, using a simple, rapid, and economical method.

Methods: For genotyping, the restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR)–amplified DNA was used and compared to allele–specific polymerase chain reaction. We genotyped 441 DNA samples obtained from the healthy general population in North–Eastern Slovenia. Genotypes for the tested group were evaluated to determine whether

Rezultati: *Rezultati, pridobljeni z alelno specifično verižno reakcijo s polimerazo, so se popolnoma ujemali z rezultati, pridobljenimi z metodo PCR/RFLP. Frekvenca alela G (0,62) je višja kot frekvenca alela A (0,38) v testirani skupini iz severovzhodne Slovenije.*

Zaključek: *Izkazalo se je, da PCR/RFLP metoda vključuje dodatne korake na račun časa analize, porabe reagentov in opreme. Na podlagi rezultatov zaključujemo, da alelno specifična verižna reakcija s polimerazo omogoča odkrivanje SNP v VKORC1 genu na bolj enostaven, hitrejši in cenovno bolj sprejemljiv način.*

the population followed the Hardy–Weinberg equilibrium. The genotypes and allele frequencies were calculated. **Results:** *The results obtained using the allele–specific polymerase chain reaction were consistent with those obtained using the PCR + RFLP method. The G allele frequency (0.62) was higher than the A allele frequency (0.38) in the general population from North–Eastern Slovenia.*

Conclusions: *The PCR+RFLP method involved additional manipulation of the PCR products at the expense of analysis time, consumption of reagents and equipment. The allele–specific polymerase chain reaction was a simple and rapid method for the detection of SNP in the VKORC1 gene, and is available in any laboratory with the minimum of equipment and reagents required.*

INTRODUCTION

Warfarin, an effective oral anticoagulant which outperforms other approved oral agents, is commonly prescribed worldwide for the treatment of deep venous thrombosis and pulmonary embolism. It is also used prophylactically for the prevention of thromboembolic events. However, over the past two decades warfarin has been underutilized as it is ranked among the top ten drugs associated with the greatest number of serious adverse drug events, due to its narrow therapeutic index and substantial inter–individual variability in dosing requirements. Warfarin therapy requires frequent monitoring of prothrombin time (PT) and international normalized ratio (INR) due to its therapeutic index. Adverse effects of warfarin include recurrence of thromboembolism and risk of bleeding (1). The management of warfarin therapy is challenging for several reasons, including the need to determine a safe and effective maintenance dose during the early phase of therapy and the fact that maintenance doses must be adjusted to compensate for changes in patients' weight, diet, disease state, concomitant use of other medications, and genetic factors (2).

Increasing evidence has demonstrated that warfarin therapy is largely influenced by demographic, clinical and genetic factors, which includes warfarin's target gene VKORC1 (3–9). The VKORC1 gene transforms vitamin K to a reduced state to function as a cofactor for the γ–glutamyl carboxylation system responsible for the modification of vitamin K–dependent proteins (7,8). In patients with decreased warfarin dose, the genotype AA as a consequence of VKORC1–1639G>A substitution is present. In such cases the warfarin dose is changed from 6.7 mg/day (GG genotype) to 2.7 mg/day (AA genotype) (10). In August 2007, the US FDA updated warfarin labeling to recommend initiating a lower warfarin dose in some patients based on VKORC1 genotypes (11). This is the first FDA recommendation to consider genetic testing when initiating a commonly prescribed medication and may set a precedent for the future use of genetic technologies in clinical practice (12).

Published methods for evaluating the VKORC1 gene involve additional manipulation of the PCR products or expensive instrumentation (13–17). The goal of our study was to detect VKORC1–1639 alleles using a simple and rapid method that is available in any laboratory with the minimum of equipment and reagents required.

MATERIALS AND METHODS

DNA samples

We genotyped 441 DNA samples obtained from the healthy general population in North-Eastern Slovenia. Genomic DNA was extracted from blood leukocytes with a simple salting-out method (18). All DNA samples were screened for the VKORC1-1639 alleles using PCR-RFLP analysis and PCR amplification with specific primers. Successful PCR amplification was confirmed by electrophoresis on 3% agarose gel, stained with SYBR Green I, and photographed for documentation.

PCR + RFLP method for identification of the VKORC1-1639 alleles

Detection of VKORC-1639 was carried out in a 15 µl reaction volume which included 1.5 µl dNTP, 1.5 µl PCR buffer, 1.5 µl MgCl2, 0.5 µl of each primer (forward and reverse), 3 µl Q additive (QIAGEN), 0.1 µl Taq DNA polymerase, 6 µl H2O and 0.5 µl human genomic DNA. The cycling conditions were 30 cycles at 94°C for 30 s, 69°C for 30 s and an extension at 72°C for 1 min. All PCRs were performed on the Biometra Thermocycler. The PCR products were digested with the HpaII restriction enzyme and incubated in a water bath overnight at 37°C prior to 3% agarose gel electrophoresis.

Table 1: *Characteristics of the allele-specific primers.*

Reaction	Primer	Nucleotide sequence 5 ,- 3'	Allele size (bp)
MIX G	VKORC1-1639-GI-F	GACCTGAAAAACAACCATTGGACG	206
	VKORC1-1639-GO-R	GCGTGAGCCACCGCAACT	
MIX A	VKORC1-1639-AI-R	GACCTGAAAAACAACCATTGGACG	238

Table 2: *Genotyping results.*

Genotype	PCR + RFLP method (N)	Allele specific PCR method (N)
GG	169	169
GA	205	205
AA	67	67
Σ	441	441

Table 3: *Hardy-Weinberg equilibrium.*

Genotype	N	Observed frequency (%)	N	Expected frequency (%)
GG	169	38,32	167,15	38,44
GA	205	46,49	208,7	47,12
AA	67	15,19	65,15	14,44
Σ	441	100	441	100

Table 4: *Frequency of allele G and A in gene VKORC1.*

VKORC1-1639	Allele number	Allele frequency
Allele G	543	0,62
Allele A	339	0,38
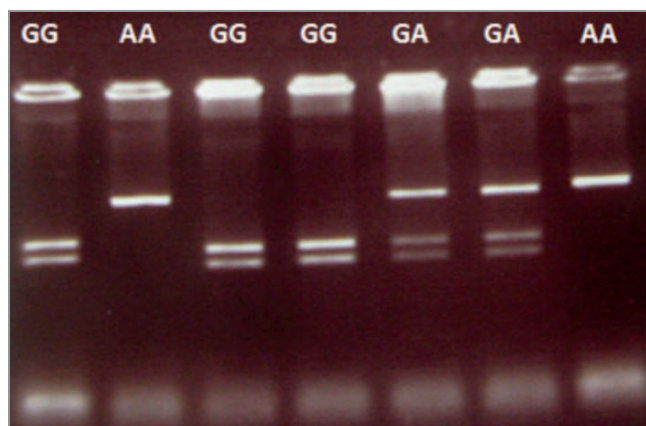
Figure 1. *3% agarose gel of RFLP results of VKORC1–1639G>A. Samples with G allele (wild–type allele) produced two bands digested by HpaII, whereas the A allele was an uncut amplicon.*

Figure 2. *3% agarose gel of allele–specific PCR product of samples from 8 subjects. (+ indicates the presence of a specific allele; – indicates the absence of a specific allele).*

The forward primer was 5'–AGGGATTCATG-CAGGGACATCTT–3'and the reverse primer was 5'–CCTCAGCCTCCCAAGTAGTTTGG–3'.

Allele–specific polymerase chain reaction
For each allele (1639G, 1639A) we performed a separate PCR reaction as previously described (19). Characteristics of the primers are shown in Table 1. PCR was performed in a total volume of 15 µl. Each reaction contained 1.5 µl of PCR buffer, 1.5 µl MgCl2, 1.0 µl of each primer (MIX G, MIX A), 3 µl Q additive (QIAGEN), 0.1 µl Taq DNA polymerase, 6 µl H2O and 0.5 µl human genomic DNA. Conditions for PCR were as follows; 30 cycles of 30 s at 94°C, 30 s at 68°C for MIX G and 65°C for MIX A and an extension of 1 min at 72°C. The reaction mixture was resolved by electrophoresis on a 3% agarose gel.
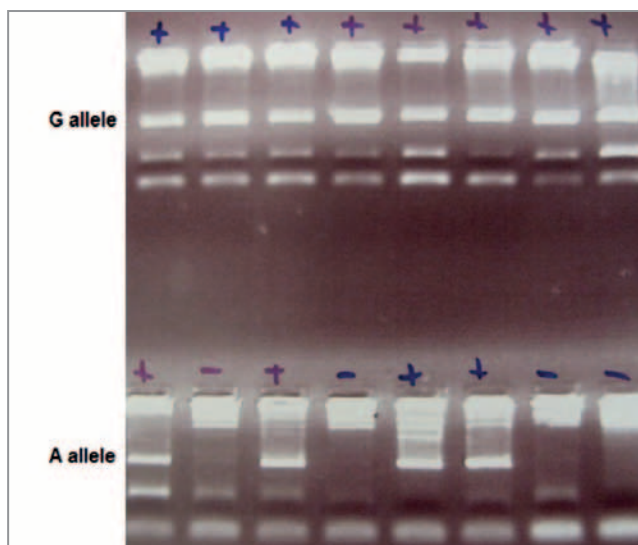
Statistical analysis
Genotypes for the tested group were evaluated to determine whether the population followed the Hardy–Weinberg equilibrium. The genotype and allele frequencies were calculated.

RESULTS AND DISCUSSION

Genotyping results using the PCR + RFLP method
We genotyped 441 DNA samples. The results derived from the PCR/RFLP reaction mixture were characterized by 3% agarose gel electrophoresis. Figure 1 shows the amplification/restriction products.

Genotyping results using the allele–specific PCR method
The same 441 DNA samples for each allele were genotyped using the allele–specific polymerase chain reaction. Results from agarose gel electrophoresis are shown in Figure 2.

PCR/RFLP vs. the allele–specific PCR method
The results obtained using the allele–specific polymerase chain reaction were consistent with those obtained using the PCR + RFLP method (Table 2).
The allele–specific polymerase chain reaction was a simple, rapid and economically acceptable method for the detection of VKORC1 polymorphism.

Statistical analysis
The expected and observed frequencies are shown in Table 3. The expected value was consistent with the observed value, thus the frequencies of genotypes did not change from generation to generation which followed the Hardy–Weinberg equilibrium. The results

are representative and can be used for further study. Table 4 presents the frequency of allele G and A in the VKORC1 gene. Allele G frequency (0.62) was higher than allele A frequency (0.38), which indicated that in this population allele G occurs more often.

CONCLUSIONS

The detection of VKORC1 gene polymorphisms was performed using 441 DNA samples obtained from a healthy general population using two molecular genetic analytical methods: the PCR–RFLP method and allele–specific polymerase chain reaction. The results obtained using allele–specific polymerase chain reaction corresponded with the results obtained using the PCR + RFLP method. It should be noted that the allele–specific PCR was a simpler, faster, and economically more acceptable method. The results were obtained within 2 hours compared to the following day with the PCR+RFLP method. The allele–specific PCR method allowed effective and accurate identification of SNP with minimum expense for equipment and reagents.

It is important to highlight that allele–specific PCR allows direct analysis of any locus of interest, and thus is generally applicable to any inherited disease provided there is sufficient sequence data. It is based on the fact that DNA polymerase, which is commonly used for PCR, lacks a 3' to 5' exonuclease activity. If a PCR primer has a mismatch at its 3'–terminal with regard to the template, the efficiency of extension by Taq polymerase will be reduced. However, the extent of the reduction in efficiency depends on the type of mismatch. In instances where the mismatch is not sufficiently refractory to extension, further deliberate mismatch near the 3' end will often deliver the specificity required (19). The main obstacle of allele–specific primer design is the determination of which additional mismatch would be introduced to obtain the required primer specificity. In our study, in order to identify the most specific primer, allele–specific primers were designed with the additional mismatch introduced at each of the positions within the four nucleotides of the 3' end. The addition of the extra mismatch, coupled with the presence of the natural mismatch at the 3' end, produced a reduction in the PCR product yield of the non–specific allele, but had an effect on the amplification of the specific allele. However, all selected primers were specific.

We conclude that the allele–specific PCR method is a simple, economical method that can be used for the rapid detection of SNP in the VKORC1 gene, and the obtained results are the basis for further research into transferring the allele–specific PCR method into clinical practice. This will help to reduce the adverse effects of warfarin, and optimize its correct initial dosage in individuals.

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