RESEARCH ARTICLE

VKORC1 gene polymorphism (-1639G>A) in warfarin therapy patients of Pakistani population

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Abstract

Objective: To observe vitamin K epoxide reductase complex subunit 1-1639 G>A polymorphism in patients resistant to warfarin therapy, and to calculate the allele frequency of the polymorphism in local patients.

Method: The cross-sectional case-control study was conducted at the Punjab Institute of Cardiology, Lahore, from 2013 to 2014 and comprised patients with heart valve replacement. Thy were divided into warfarin-resistant group 1 taking 10mg/day, 70mg/week and control group 2 taking a standard dose of 5mg/day, 35mg/week. The vitamin K epoxide reductase complex subunit 1-1639 G>A polymorphism analysis was done by polymerase chain reaction, followed by restriction fragment length polymorphism technique. Data was analysed using SPSS 20.

Results: Of the 146 patients, there were 73(50%) in each of the two groups. In group 1, there were 37(50.68%) males and 36(49.32%) were females with an overall mean age of 33±12 years, while group 2 had 36(49.32%) males and 37(50.68%) females with an overall mean age of 37±13 years. There were no significant differences in mean values of age, serum cholesterol, triglycerides and albumin levels between the groups ($p>0.05$). The G allele was the most frequently found in both groups, with 140(96%) in group-1 and 137(94%) in group-2. Overall, the homozygous GG genotype was significantly higher in the sample 132(90.4%) ($p<0.05$).

Conclusions: There was evidence found that vitamin K epoxide reductase complex subunit 1-1639 G>A polymorphism alone may not be the dominant genetic factor associated with warfarin response variability.

Keywords: Warfarin, VKORC1 gene, Single nucleotide polymorphism, PCR-RFLP. (JPMA 72: 418; 2022)

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Introduction

Thrombotic events are recognised as an important source of mortality and morbidity. Antithrombotic therapy is required for the treatment of thromboembolic events. Oral anticoagulation is widely used as antithrombotic therapy in arterial and venous thromboembolic disorders. For more than 50 years, warfarin is one of the most commonly used anticoagulants.[1] The drug metabolism in the liver is achieved by the cytochrome P450 enzymes system. The mechanism of action warfarin is vitamin K antagonism (VKA). This antagonism is achieved by inhibition of the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) enzyme complex. Inhibition of *VKORC1* results in inhibition of gamma (γ) carboxylation. The vitamin k-dependent coagulant factors II, VII, IX and X require γ carboxylation for their pro-coagulant activity. The VKAs inhibit the carboxylation of these proteins in the liver and then decrease the coagulant activity.[2]

Warfarin plays an important role by hampering the action of vitamin K-dependent factors, but a few patients do not respond to warfarin as desired. The mechanism of resistance has been implied in these situations. Warfarin resistance fails significant increase of the international normalised ratio (INR) because prothrombin time (PT) remains unaffected. So the therapeutic range of INR is not achieved by a normal dose of warfarin. Patients are considered warfarin resistant when they require warfarin >70/mg per week to maintain the INR in the target therapeutic range. Resistance of warfarin can be classified into two categories: acquired or hereditary. Another way of classifying warfarin resistance is based on its pharmacokinetics or pharmacodynamics functions.[3] The commonest causes of acquired warfarin resistance are non-compliance, decreased absorption and increased clearance of the drug from the liver, drug interactions and dietary use of food rich in vitamin K. Level of albumin in blood is another important factor resulting in the variability of warfarin dose requirement. About 50% of the protein in the plasma of a normal healthy individual is albumin. In a state of hypoalbuminaemia, the free fraction of warfarin is increased and its rate of plasma clearance is also increased.[4] In the state of hyperalbuminaemia, there will be warfarin resistance due to increased drug binding. Elevated levels of triglycerides (TG) and cholesterol in the blood also result in resistance to warfarin. Lipid emulsions may interfere with warfarin action either by increasing the production of

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clotting factors or supply of vitamin K. They also assist warfarin to bind with albumin.[5]

Hereditary warfarin resistance is caused by pharmacokinetic resistance or pharmacodynamic resistance. In pharmacokinetic resistance, genetic factors are involved which result in the rapid metabolism of the drug, whereas in pharmacodynamic resistance, the lesser activity of the drug is the cause. The gene for *VKORC1* is the main target for warfarin which encodes the target protein and affecting metabolism.[6] Studies have reported some single nucleotide polymorphisms (SNPs) that affect the drug demand to achieve the desired anticoagulant effect. Polymorphisms in *VKORC1* and Cytochrome P-450 family-2 subfamily-C polypeptide-9 (*CYP2C9*) genes have a significant role in the warfarin dose requirement. It was suggested that the *VKORC1* gene is associated with greater interindividual variability in warfarin dosing than *CYP2C9*. The *VKORC1*-1639 G>A polymorphism contributes 21% variability in the dose requirement of warfarin.[7] In a previous study, the carrier form AA genotype of the *VKORC1*-1639 gene was more likely to be hyper-responder than carrier forms GA or GG genotypes.[8]

The published data of different countries have conducted researches on the allelic frequency of the *VKORC1* gene, delineating that there are variations in different populations. These variations may be responsible for changes in the pattern of metabolism of warfarin. A research paper on the allelic frequencies in the United Kingdom revealed that the allelic frequencies of –1639G and –1639A among the British were 0.57% and 0.43%, respectively.[9] A Chinese study on allelic frequencies described that frequencies of alleles –1639G and –1639A were 0.08% and 0.9%, respectively which is significantly lower than the frequencies reported among the British. These differences in allelic frequencies among different populations might contribute to a problematic issue in warfarin dose adjustment for physicians.[10] To address the difference, the current study was planned to explore the genetic polymorphisms of the Pakistani population by examining *VKORC1* genotype profiles (1639G>A).

Materials and Methods

The cross-sectional case-control study was conducted after receiving approval from the University of Health Sciences' (UHS) Lahore's Ethical Committee for Human Research and Advanced Studies and Research Board (ASRB). The consecutive sampling of 146 patients was done from the Punjab Institute of Cardiology (PIC) Lahore between 2013 and 2014. The sample size was determined using an online formula (sample size determination in health studies version 2.0.21 WHO) with a precision level of 0.05 and a proportion of warfarin-resistant patients of 5%.[11] Patients receiving warfarin as oral anticoagulant therapy with a high risk of thrombosis were enrolled after taking written informed consent. Those taking 10mg/day warfarin (70mg/week) were considered warfarin-resistant and were placed in group 1, while those taking the standard warfarin dose of 5mg/day (35mg/week) were the controls in group 2. The main difference in the selection of cases and controls was the warfarin dose to achieve the targeted INR. As warfarin achieves its full antithrombotic effect within 5 to 7 days, so for both the groups, individuals taking warfarin for >7 days with dose adjustment to achieve the recommended INR range of 2.0-3.0 were recruited. Patients with bleeding disorders or suffering from diseases hampering vitamin K synthesis, like hepatic cirrhosis, or taking drugs affecting warfarin metabolism were excluded.

From each patient, 7ml blood sample was collected in three separate vacutainers and labelled for PT analysis, genetic analysis and analysis of albumin, TG and cholesterol levels. Genomic deoxyribonucleic acid (DNA) was isolated from peripheral blood, according to the standard phenol-chloroform method.[12] The concentration and purity of the DNA were determined by spectrophotometric analysis (ND-8000 Labtech, UK). Further, 3ml blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube (BD, United States) for DNA separation. After DNA extraction, the DNA was saved at -40°C until PCR analysis. *VKORC1* (-1639G>A) alleles identification and detection were carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The primer set for amplification of specifically required genotype was used in the following order;

VKORC1-F = 5'-GCCAGCAGGAGAGGGAAATA-3'

VKORC1-R = 5'- AGTTTGGACTACAGGTGCCT-3'

Conventional PCR experiment was performed with a total reaction volume 10µl. In each PCR tube, 10µl total reaction volume consisted of 0.5µl of DNA, 5µl of the master mix (Thermo Fisher Scientific, US), and 4.5µl of primer mix. The initial denaturation of DNA was completed at 95°C for one minute and the final denaturation was carried out for 0.05 minutes at 95°C. The temperature for primer annealing was 62°C and annealing was performed for 0.10 minutes. The extension was achieved for 0.15 minutes at 72°C and the time taken for the final extension was 3.00 minutes at 72°C. After the completion of the PCR reaction, an amplified PCR product of 290bp was resolved on 2% agarose gel (Tris-acetate [TA] and EDTA [TAE]) along with a 100bp DNA size marker. To check the presence of polymorphism in the *VKORC1* gene, 10µl of each amplified sample was digested

overnight at 37°C with a restriction enzyme derived from Moraxella species known as MspI (Thermo scientific, US). The RFLP reaction contained amplified PCR product 10µl, MspI restriction enzyme 01µl, Tango buffer 01µl and distilled water 10µl. The digestion end product was stained with ethidium bromide and analysed and on 3% agarose gel electrophoresis. The images of agarose gel were taken on a gel doc system (Bio-Rad-US).

The data were analysed using SPSS 20. Mean±standard deviation was calculated for quantitative variables, including age, cholesterol, TG and albumin. Frequencies and percentages were calculated for qualitative variables, including gender, alleles and genotypes frequencies. A Chi-square test was used to determine the association of genotypes between the two groups. The level of statistical significance was set at $p<0.05$.

Results

Of the 146 patients, there were 73(50%) in each of the two groups. In group 1, there were 37(50.68%) males and 36(49.32%) were females with an overall mean age of 33±12 years, while group 2 had 36(49.32%) males and 37(50.68%) females with an overall mean age of 37±13 years. There were no significant differences in mean values of age, serum cholesterol, TG and albumin levels between the groups (Table-1).

Table-1: Demographic and biochemical profiles of warfarin-resistant cases and normal controls.

Parameters	Warfarin resistant Cases Mean ±SD	Normal Controls Mean ±SD	*p*-value
Age (Years)	33.43±12.3	37.36±13.70	0.071
Cholesterol (g/dl)	167.1±19.1	167.45±16.25	0.910
Triglycerides(g/dl)	130.35±9.7	133.34±9.6	0.069
Albumin (g/dl)	4.34±0.49	4.44±0.55	0.258

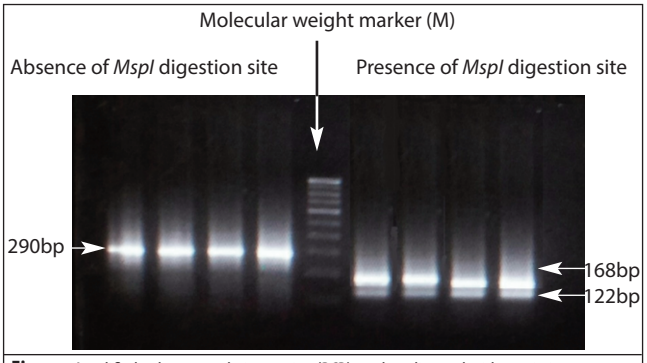

Figure: Amplified polymerase chain reaction (PCR) product digested with restriction enzyme *MspI* resolved on 2.5% agarose gel. This representative picture showed digestion of amplified PCR product on the right side with two digested fragments of 168bp and 122bp indicated the existence of homozygous G allele and on the left side absence of digestion is seen with a single undigested fragment of 290bp. The presence of a single fragment of 290bp revealed the existence of a homozygous AA allele and in the case of individuals having heterozygosity containing both G and A alleles, three fragments of 122bp, 168bp and 290bp were observed.

Table-2: Allele and genotype frequencies of vitamin K epoxide reductase complex subunit 1 *(VKORC1)* 1639 G>A in warfarin-resistant cases and normal controls.

	Warfarin Resistant Cases n (%)	Normal Controls n (%)	*p*-value
Allele			0.422
G	140 (95.89)	137 (93.83)	
A	06 (4.11)	09 (6.17)	
Total	146 (100)	146 (100)	
Genotype			0.244
GG	68 (93.2)	64 (87.7)	
GA	04 (5.5)	09 (12.3)	
AA	01 (1.4)	-	
Total	73 (100)	73 (100)	

In the *VKORC1* gene, the presence of G at position -1639 created the digestion site for restriction enzyme MspI. Whereas the presence of the A allele at the same position abolished the digestion site for the MspI enzyme (Figure).

The G allele was the most frequently found both in group 1 140(96%) and 137(94%) in group 2. The homozygous GG genotype was significantly higher in the overall sample 132(90.4%) (Table-2).

Discussion

Among oral VKAs, warfarin is the drug of choice for not only the prevention of thromboembolic disorder, but also for the treatment of these disorders. Haemorrhage is the major side effect of warfarin even in patients with low-dose requirements. Maintenance of INR in the specific window is not only dependent on the dose of warfarin, but there are many other factors on which it depends.[13] These factors can be broadly classified into inherited and acquired. The inherited factors include polymorphism of the *VKORC1* gene. This polymorphism may alter the sensitivity of warfarin to maintain the INR in the specific window.[14] Researchers have worked on this aspect and a total of 28 polymorphisms have been identified. We can broadly categorise these polymorphisms into three haplotypes of genetic variability affecting *VKORC1*.[15] Many polymorphisms have been found in the promoter region of *VKORC1*; one of such polymorphisms is SNP c.-1639G>A which accounts for the majority of the cases of the variability of response to warfarin.[16] Therefore, the present study was planned to detect the frequency distribution of *VKORC1*-1639G>A polymorphism and to express its potential role in the control of warfarin dose in Pakistani patients.

The *VKORC1*-1639G>A polymorphism examination revealed that G allelic frequency was high in both groups. In warfarin-resistant cases G allelic frequency was 95.89% while in the control group it was 93.83%. This polymorphism was also studied in various ethnic groups

in different populations of the world and it was observed that *VKORC1*-1639G allele frequency in Indian and African-American subjects was substantially higher (85% and 89%), but lower in Chinese (18%) and Malay populations (26%).[17,18] The current study's results can also be compared with another previously published data of 102 healthy northern Indians in which the allele frequency of *VKORC1*-1639G was 85.8% and GG genotype frequency was 73%.[19] However, a low allelic frequency of G was reported in Japanese and Chinese populations.[20] The difference in G allele frequency was due to genetic differences among various ethnic groups.

In the determination of inter-individual variability in dose-response of warfarin, *VKORC1*-1639 G>A genotyping is very significant.[21] Linked *VKORC1* gene variant was responsible for the variability of warfarin dose demand in different ethnic groups.[7] The requirement of the low dose of warfarin in the Japanese population was due to commonly found minor alleles (A). In the present study's results, the minor allele (*VKORC1*-1639A) frequency in the total selected population was 5.14%. It was 4.11% in warfarin-resistant cases, while in controls; it was a little higher (6.17%). Relative findings were reported by an Indian study which showing that the allelic frequency of A was 14.2%.[22] Another study in Iran also observed a 19% prevalence of the *VKORC1*-1639AA genotype.[23] However, the *VKORC1*-1639AA genotype was widely distributed in the Japanese (89.10%) and Chinese (83.70%) populations.[24,25]

In the present study, *VKORC1*-1639 homozygous GG genotype frequency was significantly higher in the total subjects. It was 90.41% ($p<0.001$). When *VKORC1*-1639 homozygous GG genotype frequency was compared amongst warfarin-resistant cases and controls, it was observed that homozygous *VKORC1*-1639 GG genotype frequency was a little higher in warfarin-resistant cases (93.2%) compared to the controls (87.7%). This minor difference was statistically non-significant ($p>0.05$). A study conducted on 112 healthy Slovak individuals reported the presence of GG, GA, and AA genotypes 73%, 52% and 12% respectively.[26] However, *VKORC1*-1639 GG genotype frequencies were the lowest in Chinese and Japanese populations.[27,28]

These findings showed that the frequency of homozygous AA genotype was related to the low warfarin dose requirement. The Chinese and Japanese populations having a high frequency of homozygous AA genotype require less warfarin dose than Indian and African populations with a high frequency of homozygous GG genotype. The present study showed that out of all the warfarin-resistant patients, only 5.5% patients were heterozygous A/G, having both the alleles. However, previous studies showed that the heterozygous A/G genotype was the highest in Romanian and Iranian populations.[20,29] In the present study, only 1.4% patients were homozygous for the *VKORC1*-1639A allele A/A, while in African-Americans its frequency was 00%[30] and in India, its frequency was 1.96%. Similarly, among the control group, 87.7% individuals were homozygous GG, 12.3% were heterozygous G/A, having both the alleles, and none was found to be homozygous AA for *VKORC1*-1639 SNP.

Table-3: Comparison of vitamin K epoxide reductase complex subunit 1 *(VKORC1)* 1639 G>A genotype and allele frequencies among different ethnic populations.

Ethnic Group	Allele Frequency (%)		Genotype Frequency (%)		
	G	A	GG	GA	AA
Pakistani population*	94.86	5.14	90.41	8.90	0.68
African American[30]	89.6	10.4	79.6	20.4	00
Saudi Arabian population[31]	57.3	42.7	37.4	39.7	22.9
Indian population[24]	85.8	14.2	73.5	24.54	1.96
Omani Patients[32]	69.6	30.4	46.9	45.3	7.7
Romanian population[33]	58.10	48.9	29.5	57.1	13.3
UK population[9]	52.6	47.4	25	56	19
Indonesian population[34]	57.3	42.7	3.7	38.5	57.8
Iranian population[7]	44.4	55.6	15.9	57.1	27
Japanese population[35]	7.5	92.5	00	15.05	84.9
Chinese population[29]	7.4	92.6	00	14.8	85.2

*: Current study.

The comparison of *VKORC1*-1639 G>A genotype and allele frequencies of the present study's findings was done with other ethnic populations (Table-3). It was determined that there is a need to study genotype frequency distribution and their effect on warfarin dose variability among different populations due to diversity in their outcomes. The introduction of direct acting oral anticoagulant (DOAC) in cardiac patients has reduced the use of warfarin in cardiac patients, and patients who have high INR and appear to be resistant to warfarin may now have the option of switching from warfarin to DOAC. Studies on DOAC in cardiac patients will give a new insight to physicians using oral anticoagulants, especially in a cardiac setting.

Further large-scale studies on the variable *VKORC1* polymorphisms in the Pakistani population are needed to validate the findings of the current study.

Conclusion

VKORC1-1639 SNP G>A alone may not be the dominant genetic factor associated with warfarin response variability in the local population. The G allele was the most frequently found allele in heart valve replacement patients. Besides, homozygous GG genotype was significantly higher in the sample.

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Study Limitation: The manuscript was completed on schedule and sent to a number of high-profile international journals. Each journal conducts its own review process. As a result, we must constantly wait until we get final input before proceeding. We recognize that the delay in publishing the current study of seven years may appear to be a constraint. Since the current study, a few more investigations on *VKORC1* polymorphisms have been published. As a result, the importance of timely publication of such investigations is emphasized.

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