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Investigation of VKORC1 Gene Polymorphism in Patients with Bleeding Complaints due to Warfarin

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Abstract

Objective: In the current study, the effect of genetic mutation was investigated on the patients referred to the emergency department due to the complaints of bleeding which receiving oral anticoagulant.

Method; The blood samples of 80 patients referred to emergency department with the complaints of bleeding who was using oral anticoagulants with any reason and of 40 healthy individuals were stored. The DNA was obtained from each sample with PCR amplification method and VKORC1 gene polymorphisms were scanned in DNA samples. Kontrol grubunda

Results: The patient and the control group were consisted of similar age and gender. On the scanning of VCOR gene, homozygote mutation was detected in 4 (5%) patients, while heterozygote mutation was defined in 51 (64%) of the cases and normal genotype were in 25 (31%) cases in patient group. Heterozygote mutation was found at 28 (70%) individual and normal genotype were detected at 12 (30%) individual in control group.

Conclusion: Warfarin is one of the important treatment modalities for the antithrombotic therapy in the thrombotic events. However, laboratory follow-up requirement for bleeding, requires a serious follow-up during the use. Clarification of the genetic basis of the

relationship between bleeding and dose setting will be increased the quality of life in the patients that treated with antithrombotic agents.

Key words: Warfarin, VCOR gene polymorphism, bleeding

INTRODUCTION

Warfarin is the most commonly used oral anticoagulant in the world. Although this agent is indispensable for treatment of thromboembolism, it is not so easy to adjust the appropriate dose to each patient due to the large inter-individual variation in the requirement for this drug. An insufficient dose will result in failure to prevent thrombosis, while overdose increases the risk of unexpected bleeding. The maintenance dose of warfarin is usually determined by monitoring prothrombin time using an international normalization ratio (INR). An INR of 1.5–2.5 is recommended for Asian populations (1).

Warfarin is a widely used coumarin anticoagulant prescribed for patients with venous thrombosis and pulmonary embolism, chronic atrial fibrillation and prosthetic heart valves. Interindividual differences in drug response, a narrow therapeutic range and the risk of bleeding, all make warfarin a difficult drug to use clinically. Warfarin dose requirements, the stability of anticoagulation and risk of bleeding are influenced by environmental factors such as the intake of vitamin K, illness, age, gender, concurrent medication and body surface area, and by genetic variation (2-9). To be able to improve the benefit–harm profile associatedwith warfarin therapy, all these factors need to be taken into account.The efficacy of warfarin and other vitamin K antagonists in preventing and treating thrombosis has been well demonstrated in numerous randomized controlled trials and metaanalyses (10).

Providing of the appropriate therapy range with the different doses in different persons and different bleeding frequency for each case have been directed the researchers to reveal the ethiology. The idea of the genetic ground may be effective on the dose setting and change of bleeding frequency has been gradually come into prominence and the studies conducted on this topic have been started to take place in the literature (11,12).

Objective of this study is to define the genetic mutations among the patients referred to our ED with the complaints of bleeding and to rewiev the literatural knowledge indicating prevention of the fatal complications such as the bleeding by starting of the warfarin treatment according to the genetic mutation.

MATERIAL & METHODS

The blood samples of 80 patients using oral anticoagulant and referred to the emergency department with a bleeding diathesis with any reason were stored and studied. The patients included to this study were assessed in terms of age, gender, reason of referral to ED, reason and duration of warfarine use, PT, PTT and INR values and polymorphism of VCOR. The results were statistically analyzed. The ethical approval was taken fort he study.

Total genomic DNA was extracted from the 200µl pheripheric blood samples by the Nucleospin blood (250) DNA isolation procedure (Macherey - Nagel, Germany). Regions emcompassing VKORC1 -1639G>A mutation was simultaneously in vitro amplified and biotin-labelled in a single (multiplex) amplification reaction (Vienna Lab, PGX-Trombo StripAssay, Austria). PCR was performed in a Perkin Elmer 9600 and the profile consisted of an initial melting step of 2 minutes at 94°C; followed by 35 cycles of 15 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C; and a final elongation step of 3 minutes at 72°C. The mutation analysis was performed by StripAssay technique (Vienna Lab, PGX-Trombo

StripAssay GmbH, Austria) which is based on the reverse-hybridization principle automatically. The normal, heterozygous and homozygous mutant/ non-mutant genotype profiles of each gene was determined using the enclosed CollectorTM sheet.

Statistical Analysis

The continuous variables were expressed as mean ± SD. The categorical variables were stated as percentages. The independent parameters were compared using Student's t test for two independent groups and ANOVA followed Bonferroni Post Hoc test or Kruskal-Wallis test for three independent groups. The evaluation of data were performed by using SPSS 15.0 (SPSS, Inc., Chicago, Illinois) software. Two sided hypotesis were considered and if $p \leq 0.05$ were considered significant.

RESULTS

Eighty patients who were referred to our emergency department and 40 healthy persons were included in the current study. Characteristics of the patient group according to the genotypes (Table1), the results of comparison of the bleeding severity in the patient group according to genotypes (Table 2), comparison of the groups in terms of A allel presence (Table 3) and comparison of the bleeding severity in terms of the A allel presence (Table 4) were presented.

Table 1: Characteristics of the patient group according to the genotypes

	CC (Normal) (n:25)	CT (Heterozygous)(n:51)	TT (Homozygous) (n:4)	p
Age (years±SD)	59±14 (median 55)	61±11 (median 63)	60±7 (median 63)	0.851
Gender,female (n,%)	16(64%)	27(53%)	3(75%)	0.505
Hypertension (n,%)	13(52%)	31(61%)	3(75%)	0.609
Diabetes mellitus (n,%)	7(28%)	16(31%)	1(25%)	0.932
Hyperlipidemia (n,%)	11(44%)	16(31%)	1(25%)	0.506
Smoking (n,%)	10(40%)	21(41%)	1(25%)	0.817
Disease duration (years±SD)	10±11 (median 8)	13±13 (median 9)	9±3 (median 8.5)	0.657
Warfarin doze (mg)	4.6±1.5 (median 5)	4.5±1.0 (median 5)	3.1±1.2 (median 2.5)	0.087
PT	55±47 (median 39)	70±44 (median 64)	84±67 (median 86)	0.243
PTT	49±21 (median 45)	50±26 (median 42)	65±50 (median 44)	0.892
INR*	3.4±3.8 (median 1.7)	5.5±3.7 (median 5.1)	7.4±6.0 (median 6.8)	0.003
BUN	34±8 (median 34)	34±10 (median 33)	26±3 (median 26)	0.157
Kreatinin	1.2±0.4 (median 1.3)	1.2±0.5 (median 1.2)	1.0±0.3 (median 1.0)	0.622
ALT*	33±16 (median 32)	44±19 (median 44)	57±13 (median 60)	0.009
AST*	41±20 (median 39)	56±22 (median 51)	66±17 (median 70)	0.014
Antiplatelet agents n(%)	5(20%)	7(14%)	1(25%)	0.697
B blockers n(%)	17(68%)	37(73%)	2(50%)	0.617
Statins n(%)	11(44%)	16(31%)	1(25%)	0.506
ACE inh/ARB n(%)	13(52%)	26(51%)	1(25%)	0.589

* The mean of three groups were analyzed by using ANOVA followed Bonferroni Post Hoc test. Three means were significantly difference each other ($p<0.001$).

Table 2: Comparison of the bleeding severity in the patient group according to genotypes

	CC (Wild Type)	CT (Heterozygous)	TT (Homozygous)	p
Pre-bleeding hemoglobin (gr/dL)	11.9±1.8 (median 12.5)	11.6±1.7 (median 11.4)	10.8±0.5 (median 10.7)	0.560
Post-bleeding hemoglobin (gr/dL)	9.8±1.8 (median 10.1)	9.3±1.6 (median 9.5)	8.1±0.5 (median 8.0)	0.112
*Hb change percentage (%)	17±4 (median 16.9)	20±5 (median 19.9)	25±5 (median 26.7)	0.017

* The mean of three groups were analyzed by using ANOVA followed Bonferroni Post Hoc test. Three means were significantly difference each other (p<0.001).

Table 3: Comparison of the groups in terms of A allel presence

	Group without A allel (n:25)	Group with A allel (n:55)	p
Age (years±SD)	59±12 (median 55)	60±11 (median 62)	0.589
Gender,female (n,%)	16(64%)	30(55%)	0.293
Hypertension (n,%)	13(52%)	34(62%)	0.279
Diabetes mellitus (n,%)	7(28%)	17(31%)	0.505
Hyperlipidemia (n,%)	11(44%)	11(31%)	0.188
Smoking (n,%)	10(40%)	22(40%)	0.595
Disease duration (years±SD)	10±11 (median 8)	13±13 (median 9)	0.371
Warfarin doze (mg)	4.6±1.5 (median 5)	4.4±1.1 (median 5)	0.568
PT	55±47 (median 39)	71±46 (median 64)	0.093
PTT	49±21 (median 45)	51±28 (median 42)	0.803
INR	3.4±3.8 (median 1.7)	5.6±3.8 (median 5.1)	0.001
BUN	34±8 (median 34)	34±10 (median 33)	0.799
Kreatinin	1.2±0.4 (median 1.3)	1.2±0.5 (median 1.1)	0.771
ALT	33±16 (median 32)	45±19 (median 44)	0.007

Continued to Table 3:

AST	41±20 (median 39)	57±22 (median 54)	0.006
Antiplatelet agents n(%)	5(20%)	8(15%)	0.378
B blockers n(%)	17(68%)	39(71%)	0.495
Statins n(%)	11(44%)	17(31%)	0.188
ACE inh/ARB n(%)	13(52%)	27(49%)	0.500

Table 4: Comparison of the bleeding severity in terms of the A allel presence

	Group without A allel (n:25)	Group with A allel (n:55)	p
Pre-bleeding hemoglobin (gr/dL)	11.9±1.9 (median 12.5)	11.5±1.6 (median 11.2)	0.358
Post-bleeding hemoglobin (gr/dL)	9.8±1.8(median 10.1)	9.2±1.6 (median 9.2)	0.114
Hb change percentage (%)	17±4 (median 16.9)	20±5 (median 19.6)	0.023

On the genetic examination, homozygote mutation was found in VCOR gene of 4 (5%) 4 cases, heterozygote mutation in 51 (64%) cases and normal genotype in 25 (31%) cases. Whereas, homozygote mutation was not defined in any control. Heterozygote mutation was found in 28 (70%) cases and normal genotype in 12 (30%) cases from the control group (Table 5).

Table 5: Distribution of the patient and control groups in terms of VCOR genotypes

Group	**CC (Wild Type)**	**CT (Heterozygous)**	**TT (Homozygous)**	**Total**
Patient group (n,%)	25(31%)	51(64%)	4(5%)	80(100%)
Control group (n,%)	12(30%)	28(70%)	0(0%)	40(100%)

When allel frequencies were compared, G allel frequency was detected in 63% and A allel frequency in 37% of the patient group. Whereas G allel frequency was found in 65% and A allel frequency in 35% of the patient group (Table 6).

Table 6: Distribution of Allel frequencies in the patient and control groups

Group	G Allel	A Allel	Total
Patient group (n,%)	101(63%)	59(37%)	160(100%)
Control group (n,%)	52(65%)	28(35%)	80(100%)

Of the patients, 28% (n=22) were using warfarine due to deep vein thrombosis, 21% (n=17) due to pulmonary thromboembolism, 19% (n=15) for atrial fibrillation, 10% (n=8) for cerebrovascular shock and 23% (n=18) due to mitral valve replacement.

DISCUSSION

When dosed properly, warfarin has been shown to decrease morbidity and mortality rates in several common ailments including deep venous thrombosis, pulmonary embolism, atrial fibrillation, and cardiac valve replacement. Unfortunately, this benefit must be weighed against warfarin's significant annual risk of minor or major bleeding (10%), intracranial hemorrhage (1%), and death (0.6%) (13). The risk of warfarin related bleeding is highest at the start of anticoagulant therapy. This risk to be 10 times higher at the outset of therapy as opposed to at the 1 -year mark of use (13). Bleeding events are most likely to occur within the first 90 days of therapy, but the incidence never falls to zero. The risk of bleeding is higher when INR is over 3, but bleeding can also occur when the INR is within the therapeutic range (14).

The major predisposing factors for warfarin-related bleeding can be classified into four broad categories: intensity of anticoagulant therapy, patient characteristics, duration of therapy, and the concomitant use of interacting medications (15). Warfarin has a narrow therapeutic index and thus the dose required to achieve therapeutic anticoagulation is very close to the dose that leads to over-anticoagulation. This unpredictability leads to difficulties in maintaining patients within a therapeutic anticoagulation range, which usually is an international normalized ratio (INR) of 2.0–3.0 (10).

High rates of adverse events during warfarin initiation, knowledge of a patient's warfarin sensitivity before initiation of therapy could prevent negative therapeutic outcomes (e.g., major bleeding, thrombosis) (16). The most important genes affecting the pharmacokinetic and pharmacodynamic parameters of warfarin is VKORC1 (vitamin K epoxide reductase complex subunit 1) (10). Previous studies have evaluated the utility of genotyping for VKORC1 in predicting warfarin dose in patients on longterm anticoagulation, but have found that these genotypes account for only about 60% of variation in dose (17). These two genes, together with environmental factors, partly explain the interindividual variation in warfarin dose requirements (18).

Polymorphisms in the VKORC1 gene and a limited subset of environmental determinants account for around 50–60% of the variance in warfarin dose requirement (18-24).

Warfarin exerts its mechanism of action by binding to vitamin K epoxide reductase complex subunit 1 (VKORC1) and inhibiting its activity. VKORC1 activity is necessary to regenerate reduced vitamin K, which is necessary for the production of functional vitamin-Kdependent clotting factors (factors II, VII, IX and X) and anticoagulant proteins (proteins C, S, and Z) (25).

The gene of the major protein component of VKOR is found in the complex subunit 1, known as VKORC1. Mutations in this gene have been associated with a deficiency in vitamin-K-dependent clotting factors, resulting in increased sensitivity to warfarin or warfarin resistance or insensitivity (18,26). The interesting questions are whether this will lead to a recommendation for genotyping in the label for warfarin, and if this would change clinical practice and, more importantly, improve the use and safety of warfarin (10).

Yuan et al.(27) recently reported their findings of a novel VKORC1 whereby the AA genotype was present in all warfarin-sensitive Chinese patients. Interethnic comparisons revealed the frequency of the homozygous AA genotype to be lower in Caucasians (14%) than Chinese (79.7%) populations.

Major risk for bleeding due to Warfarine increases with the presence of other risks. These risk factors can be summarized with the abbrevation of COUMARINS. COUMARINS stands for Chronic renal insufficiency, Other drugs, Uncontrolled hypertension, Malignancy, Alcohol abuse, Rebleeding risk, Increased age, Neuropsychiatric-physical impairment and Stroke (23-26).

Although bleeding can occur with any level of anticoagulation, use of warfarin for various indications have consistently shown that higher levels of anticoagulation are associated with higher rates of bleeding. It is even believed intensity of anticoagulation to be the single biggest determinant of a patient's bleeding risk (28-30).

Yet, the overall bleeding risk is not necessarily linear. In fact, the bleeding risk increases exponentially as the INR surpasses 5.0 in otherwise healthy persons and 3.0 in older patients and those with a history of cerebrovascular disease (31).

In the current study, homozygote mutation was found to cause higher INR, ALT and AST values and statistically higher percentage of Hb change despite lower Warfarin doses in the patient group ($p<0.05$). Also it was defined in the patient group that presence of A allel leads to INR, ALT and AST values and a statistically significant decrease in Hb percentage compared to the group without A allel and that the bleeding was in more serious dimensions ($p<0.05$).

Because of the complications developing during the treatment, before making a decision to discontinue or continue the treatment, the risk of the rebleeding in case of continuation of the treatment and development of thromboembolism in case of continuation should be taken into account. However, risk for the thromboembolism is much lower than the rebleeding risk (28).

In conclusion, definition of the detoxification profile in the patients required warfarine therapy is excitatory about the warfarine dose and the bleeding which is the most frequent and important complication can be avoided to reach life threatening dimensions.

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