ARAŞTIRMA YAZILARI **ORIGINAL ARTICLE**

ASSOCIATION BETWEEN CYP2C9 GENETIC POLYMORPHISM AND WARFARIN TREATMENT

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SUMMARY

Background: The aim of this study was to evaluate the genetic variants of warfarin used on individuals

Methods: Two separate groups were tested in accordance to mean daily warfarin dosage (Group 1: ≥ 5 mg/day and group 2: < 5mg/day). Genotyping of CYP2C9*2 and CYP2C9*3 allelic variants was carried out by PCR and subsequent Avall or Nsil digestion, respectively.

Results: Frequency of CYP2C9 *1 *1 genotype was found higher in patients who were taken warfarin above 5 mg/day. Statistically association was found between warfarin dose and both CYP2C9 *1 *1 and CYP2C9 *1 *3 polymorphisms. Mean INR values were lower in CYP2C9*1 *1 genotype. Also mean INR values of complicated patients group were higher than non-complicated group. No relation was found between genotype polymorphisms and outcome of complications. Higher INR values were found as a significant risk factor for reveal of the complications.

Discussion: To know genetic polymorphisms before the warfarin treatment, will obtain pioneer knowledges for drug dosage. So this marker could be a useful pre-treatment marker for prevents to reveal complications.

CYP2C9 GENETİK POLİMORFİZMİ İLE WARFARİN TEDAVİSİ ARASINDAKİ İLİŞKİ

Amaç: Bu çalışmanın amacı, warfarin kullanan hastalarda genetik varyasyonun araştırılmasıdır.

Metod: Ortalama günlük warfarin dozlarına göre 2 farklı grup çalışmaya alındı (Grup 1: ≥ 5 mg/gün ve group 2: < 5mg/gün). CYP2C9*2 ve CYP2C9*3 varyantlarını tesbit etmek amacı ile sırasıyla polimeraz zincir reaksiyon tekniği ve ''Avall or Nsil digestion'' yöntemi kullanıldı.

Bulgular: Günlük warfarin dozu 5 mg üzerinde olan hastaların CYP2C9 *1 *1 genotip sıklığı daha fazla bulundu. CYP2C9 *1 *1 ve CYP2C9 *1 *3 polimorfizmleri ile warfarin dozları arasında statistiksel ilişki bulundu. Ortalama INR değerleri CYP2C9*1 *1 genotipinde daha düşük bulundu. Komplikasyon çıkan hasta grubunda, non-komplike hasta grubuna göre ortalama INR değerleri daha yüksek bulundu. Genotip polimorfizm ile komplikasyonun ortaya çıkması arasında hiçbir ilişki bulunmazken önemli risk faktörleri olarak yüksek INR değerleri bulundu.

Sonuç: Tedaviden önce genetik polimorfizmin bilinmesi olası ilaç dozları hakkında ön bilgi sağlayabilir ve böylece gelişebilecek komplikasyonları engellemek amacı ile öncül bir markır olarak kullanılabilir.

INTRODUCTION:

Anticoagulation with warfarin, a vitamin K antagonist, has been long established in the management of different diseases and has been demonstrated that it can reduce the risk of ischaemic stroke for high-risk patients such as atrial fibrillation [1]. In this respect, warfarin is the most recommended anticoagulant in the world and also in Turkey.

The dose of this drug is usually adjusted according to regular determinations of the internationally normalized ratio (INR) but a large interindividual variability in the dose-response relationship exists. Variability may be one cause of the bleeding complications or insufficient anticoagulation. The dose-response variations might partly be due to differences in pharmacokinetics of the drug [2]. In addition the differences in warfarin response may be due to intake of vitamin K, ethnicity, illness, adherence, age, gender, concurrent medications, lipid profile and body mass index (BMI) [1,3,4].

In recent years attention has been focused on possible genetic determinants especially on CYP2C9, which is an isoform of cytochrome P450 enzyme. It is involved in the metabolism of the most of the drugs used in the treatment of thromboembolic disorders. Genetic variations may be of more practical significance and importance

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in optimising drug therapy [5,6]. Retrospective clinical studies have demonstrated that individuals with mutant allelic variants of CYP2C9 gene slowly metabolise warfarin and may be at increased risk of haemorrhage during initiation of therapy [7].

The aim of this study was to determine the genetic variants of warfarin used on individuals and to evaluate the significant risk factors for drug dose management and revelation of complications.

MATERIAL AND METHODS:

Selection of Patients

Sixty patients (36 women / 24 men; mean age: 61 ± 13.1 years; 29 to 82 years) who were receiving warfarin for different diagnosis were recruited from the Neurology and Internal Medicine clinics. They had been taking same daily dose of warfarin for at least 1 month before samples were collected [8] and any medication had not been added in the last two months to their prior therapy. Patients were separated into 2 groups according to mean daily warfarin dosage (warfarin dosage for ≥ 5 mg/day in group 1, dosage for < 5mg/day in group 2). The rate of complication for each group was determined. Patients were excluded when there was a non-genetic explanation for altered warfarin requirements such as liver disease, alcohol consumption, underweight (BMI<18 kg/ m2), a diet rich in green leafy vegetables including broccoli, spinach, kale, or cauliflower (>2 servings/ day).

DNA Genotyping

Genomic DNA was isolated from the peripheral blood samples anticoagulated with EDTA according to a standard protocol [9]. For detection of the CYP2C9*2 and CYP2C9*3 variants a protocol based on a polymerise chain reaction (PCR) technique and endonuclease digestion was used [10]. PCR reaction was performed in a total volume of 25 µl containing approximately 100 ng DNA, 2.5 µl of 10X polymerise buffer, 2.0 mmol/l MgCl2, 0.2 mmol/L dNTPs, 0.4 µmol/l of each primer, and 1 U of Taq polymerise (Fermentas). The PCR program on PTC-150 MinicyclerTM (MJ Research) thermal cycler was as follows: an initial denaturation step at 94 ºC for 4 min, followed by 33 cycles of 45 sec at 94 ºC, 30 sec at 57 ºC, 45 sec at 72 ºC, and a final extension step of 8 min at 72 ºC.

For CYP2C9*2 (Arg144Cys) the following primers were used: CYP2C9–2 F: 5′ – TAC AAA TAC AAT GAA AAT ATC ATG – 3′ and CYP2C9-2 R: 5′ – CTA ACA ACC AGA CTC ATA ATG – 3′. After amplification, 10 µL of the 691 bp PCR product was digested overnight at 37°C with 5U of the AvaII restriction enzyme [10]. The digested product was analysed in a 2% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. Samples with Arg144 had a single 691 bp band, while samples with Cys144 gave 527 and 164 bp bands.

For CYP2C9*3 (Ile359Leu) the following primers were used: CYP2C9–3F: 5′ – AAT AAT AAT ATG CAC GAG GTC CAG AGA TGC – 3′ and CYP2C9-3R: 5′ – GAT ACT ATG AAT TTG GGA CTT C – 3′. An aliquot of 10 µL of the 141 bp PCR product was digested overnight at 37°C with 5U of the NsiI restriction enzyme [10]. The digested product was analysed in a 4% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. Samples with Ile359 had 112 and 29-bp bands, while samples with Leu359 gave 141 bp band (Fig. 1).

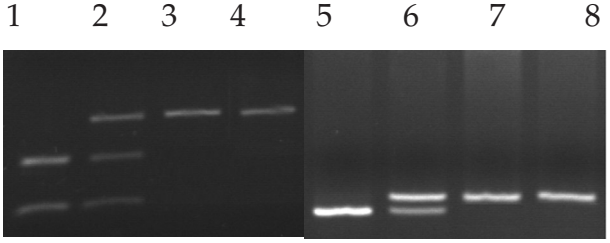

Figure 1. Agarose gel electrophoresis showing the CYP2C9*2 (a) and CYP2C9*3 (b) genotyping. Lane 1: Cys144/ Cys144; lane 2: Cys144/ Arg144; lane 3: Arg144/ Arg144; lane 4: CYP2C9*2 PCR product; lane 5: Ile359/ Ile359; lane 6: Ile359/ Leu359; lane 7: Leu359/ Leu359; lane 8: CYP2C9*3 PCR product.

Statistical Analysis:

The statistical package SPSS for windows (release 10.0) were used for data analysis. Numerical values were reported as mean±SD or as a proportion of the sample size. Comparisons between the study and control groups were made with the X_ test for categorical data's and Mann Whitney U test for continuous data's. Logistic regression analysis was done to predict the risk factors. A p value< 0.05 was accepted as statistically significant.

RESULTS

The duration of warfarin use for all the patients (n=60) was 30.7± 6 months. The primer indication for anticoagulation treatment was mitral valve replacement in 11 patients (18.3 %) and the second was cerebral infarction in 10 patients (16.6 %). Patient demographics including sex and age and the kinds of complications for two groups were recorded in table 1 and 2 with no statistically difference. Mean INR values of complicated patients group were found higher than non-complicated group (5.59 for complicated patients and 2.59 for non-complicated patients group, with Mann-Whitney U test; p=0,003).

The findings of genetic polymorphism of all and complicated patients were summarized in table 3 and table 4.

No patients had CYP2C9 * 2 * 2 and CYP2C9 * 2 * 3 genotypes in group 1. Frequency of CYP2C9 *1 *1 genotype was found higher in patients who took warfarin above 5 mg (with x2 test; p= 0.025) (see table 3).

The mean daily warfarin dose was found to be 5.07 milligrams for CYP2C9 *1 * 1; 3.77 milligrams for CYP2C9 *1 * 2; 3.31 milligrams for CYP2C9 *1 * 3; 3.75 milligrams for CYP2C9 *2 * 2 and 2. 82 milligrams for CYP2C9 * 2 * 3. The patients with CYP2C9 *1 *1 and CYP2C9 *1 *3 polymorphisms were found to be using higher dose of warfarin (with Mann Whitney U test p = 0.001 and p= 0.023, respectively).

The relation between mean INR values and genotypes were summarized in table 5. Mean INR values were found lower in CYP2C9*1 *1 genotype (with Mann Whitney U test; p= 0.031).

There was no relation between genotype polymorphisms and outcome of complications (see table 4).

Higher INR values were found as a significant risk factor for reveal of the complications (with Logistic regression analysis; p= 0.003).

Table 1: Patient demographics, INR values and complication rates

	Group 1 (Warfarin dose ≥ 5 mgr) (n=27)	Group 2 (Warfarin dose ≥ 5 mgr) (n=33)	P
Sex (F/M)	19/8	17 / 16	NS
Age (y; mean±SD)	60.70 ± 13.5	61.3 ± 12.9	NS
INR (mean ±SD)	3.54 ± 4.02	3.63 ± 3.4	NS
Complicated patients (no; %)	7 (25.9%)	13 (39.3%)	NS

Table 2 : Complications of two groups.

	Group 1 (Warfarin dose ≥ 5 mgr) (n=27)	Group 2 (Warfarin dose ≥ 5 mgr) (n=33)	P
Echymosis	3 (11,1%)	8 (24.2 %)	NS
Epistaxis	1 (3.7 %)	4 (12.1%)	NS
Hematuria	1 (3.7%)	3 (9.1%)	NS
Femoral haematoma	2 (7.4%)	2 (6.1%)	NS
Menoragia	1 (3.7%)	1 (3.0%)	NS
Gingival haemorrhage	1 (3.7%)	1 (3.0%)	NS
Major complication	4 (14.8%)	3 (9.1%)	NS
Minor complication	3 (11.1%)	10 (30.3%)	NS

Table 3 : Genetic polymorphism of two groups

	CYP2C9 *1* 1	CYP2C9 * 1 * 2	CYP2C9 * 1*3	CYP 2C9* 2 *2	CYP2C9*2 *3
Group 1	20 (74.1%)	4 (14.8 %)	3 (11.1 %)	0	0
Group 2	15 (45.5 %)	6 (18.2%)	9 (27.3%)	1(3%)	2 (6.9 %)
Total	35 (58.3%)	10 (16.7 %)	12 (20%)	1 (1.7 %)	2 (3.3 %)
P	0.025	NS	NS	NS	NS

Table 4 : Genetic polymorphisms of the complicated patients

	CYP2C9 *1* 1	CYP2C9 * 1 * 2	CYP2C9 * 1*3	CYP 2C9* 2 *2	CYP2C9*2 *3
Group 1	4 (57.1%)	2 (28.6 %)	1 (14.3 %)	0	0
Group 2	5 (38.5%)	1 (7.7%)	5 (38.5%)	1 (7.7%)	1 (7.7%)
Total	9 (45%)	3 (15 %)	6 (30%)	1 (5%)	1 (5%)
P	NS	NS	NS	NS	NS

Table 5: Mean INR values according to genetic polymorphisms

	CYP2C9 *1* 1	CYP2C9 * 1 * 2	CYP2C9 * 1*3	CYP 2C9* 2 *2	CYP2C9*2 *3
Mean INR Values	2.99±3.17	5.69±6.16	3.59±1.95	4.1	3.27±3.05
P	0.031	NS	NS	NS	NS

DISCUSSION

Warfarin exerts its anticoagulant activity by inhibiting the activation of vitamin K- dependent blood clothing proteins including factors II, VII, IX and X, which are required for normal haemostasis [1]. The genetic determinants CYP2C9 is almost exclusively involved in the metabolism of the S-enantiomers of warfarin through 6-and 7-hydroxylations by the cytochrome P450 system of isozymes [3, 11]. The found frequency of CYP2C9 in a Turkish population of 449 subjects were 61.72% for CYP2C9* 1* 1; 18.04 % for CYP2C9 * 1 * 2; 17.23 % for CYP2C9 * 1* 3, 1.00 % for CYP2C9 *2 *2; 1.10 % for CYP2C9 * 2 * 3 and 0.80 % for

CYP2C9 * 3 * 3 [12]. In our study, frequencies were 45 % for CYP2C9* 1* 1; 15 % for CYP2C9 * 1 * 2; 30 % for CYP2C9 * 1* 3, 5% for CYP2C9 *2 *2; 5% for CYP2C9 * 2 * 3. We have no patient with CYP2C9 * 3 * 3 allele. The probable cause of difference might be that our group was a small study group.

In previous studies, the maintenance dose of warfarin was found in a significant relation with genotypes CYP2C9 *2 and *CYP2C9 *3 and have demonstrated that patients carrying CYP2C9 *2 and *3 allele variants need reduced maintenance dose, show a longer induction period and have a higher risk of bleeding [13].

Higashi et al [14] assessed the influence of genetic variants on anticoagulated related outcomes with warfarin therapy and the maintenance doses of warfarin were found to be the highest (5.63 milligrams) for CYP2C9*1 *1 while CYP2C9 * 3 * 3 needed the lowest dose. Researchers concluded that the mean maintenance dose has a significant relation to genotype. Tabrizi et al [15] found that patients with wild type (CYP2C9 *1*1) needed a mean weekly warfarin dosage of 40.1 milligrams while patients with the genetic variant CYP2C9 *2 needed lower and with CYP2C9 * 3 needed the lowest dose. Taube et al's [16] group's mean daily dose for the wild type allele was 5.01 milligrams. A significant relation between average maintenance dose and genotype, specifically with homozygous CYP2C9 *2 and heterozygous *3 was observed. Lowest mean warfarin dose was related with homozygous * 2* 2 allele. No patients with the CYP2C9 *3*3 polymorphism were identified. The authors concluded that patients stabilized on a maintenance dose of warfarin with variations were not at increased risk of bleeding because they were maintained within their therapeutic INR.

In our study a significant relation between average maintenance dose and genotype, with CYP2C9 *1 *1 and CYP2C9 *1 *3 was observed.

Higashi et al [14] found a relation between genotype and increased INR. Aithal et al [17] studied the association of polymorphisms and the risk of bleeding and found no clinical difference in the incidence of minor bleeding episodes among the groups but there was a significant difference in major bleeding episodes. They concluded that the variant alleles affect the dosing of warfarin upon initiation.

According to Joffe et al [18] CYP2C9 polymorphisms do not increase bleeding rates and suggest that CYP2C9 *2 and *3 polymorphisms are highly prevalent in patients requiring low warfarin doses.

In a recent study including a review and meta-analysis, of 11 studies with 3029 patients' were identified [19]. Patients with CYP2C9*2 and CYP2C9*3 alleles were found to have lower mean daily warfarin doses and a greater risk of bleeding. The authors concluded that evidence for the clinical utility and cost-effectiveness of genotyping is needed before routine testing can be recommended.

In our study, mean INR values were found lower in CYP2C9*1*1 genotype but we couldn't find any relation between genotype polymorphisms and outcome of complications. Besides higher INR values were found as a significant risk factor for reveal of the complications.

CONCLUSION:

In the future, genetic polymorphism could be an useful pre-treatment examination for prevent to reveal complications earlier.

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