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Investigation of the *CYP2C9* Gene Polymorphism Frequency and its Effects on the Warfarin Dose Used by the Patients in a Population from Iran

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ABSTRACT

Warfarin is an anticoagulant drug that is used to treat and prevent thromboembolic disorders. Despite its efficacy, this substance has a therapeutic index, and the response to it is completely variable. Plenty of this drug leads to bleeding, and a low dose of it leads to poor blood coagulation. The aim of this study was to survey the relationship between the dose of warfarin usage and Gene polymorphism and its relation with age and sex of the patient. Blood sampling was done intravenously by receiving consent forms from a total of 100 people with heart disease who were referred to a medical laboratory in the city of Kerman to perform the PT Test. After DNA extraction, the PCR_RFLP method was done on all samples. The frequency of *the CYP2C9 gene polymorphism and the relationship with the dose of warfarin usage*, *age*, and gender of patients has been investigated. The frequency of observed genotypes was 1%, 12%, 87%, sequentially CC, TC, and TT, in the present study. There was a meaningful relationship between age and the dose of warfarin usage (P=0.0001). Data analysis showed that there was no meaningful relationship between variant genotypes *CYP2C9* and the dose of usage of warfarin (P=0.999), and also male and female groups in terms of genotype polymorphism (P=0.115). Based on the results, it seems that other genes interfere with the usage of warfarin according to the genotype of each population.

Keywords: CYP2C9, Gene Polymorphism, PCR-RFLP, Vitamin K, Warfarin.

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INTRODUCTION

Warfarin is the most common oral anticoagulant for the prevention and Treatment of thrombotic diseases, such as atrial fibrillation, and the risk of thrombosis [1-3]. It's important to prescribe a suitable dose of warfarin for each patient because the lack of amounts of patients needed dose, results in the occurrence of life-threatening thrombosis, and the excess amounts will result in heavy bleeding [1, 2]. Cytochrome P450 is one of the hepatic enzymes that is needed for the metabolism of important physiologic drugs such as warfarin [4]. The PT_INR test was used to monitor the dose of warfarin. It is necessary to minimize the side effects of warfarin and achieve the best therapeutic dose and maintain INR in the range of 2_3 in thrombotic diseases. According to most guidelines, to achieve the target INR, especially in the first weeks of treatment, it is necessary to prescribe a maintenance dose of warfarin (approximately 5 mg). In addition to demographic characteristics such as age, sex, weight, and diet patient, genetic polymorphisms also play a crucial role in the patient's response to medication [1, 5]. Some studies have shown that several genes are effective in metabolizing warfarin. One of these genes is CYP2C9, which is located on the long arm of chromosome 10. The product of this gene is a protein with an enzyme called cytochrome p 450 2c9 under the influence of this enzyme. Warfarin is converted to its inactive metabolites, 6-hydroxy s-warfarin and 7-hydroxi s-warfarin by, which decreases its plasma level. There are several polymorphisms in the *CYP2C9* gene that affect the activity of the metabolizing enzymes of warfarin. The variation in the *CYP2C9* gene in people is relatively significant, while it is rarely seen in people of African and Asian origin [6, 7].

MATERIALS AND METHODS

After receiving the consent form, blood sampling was done on a hundred people who have heart disease and were referred to a medical laboratory in Kerman city for testing PT (in the period from May to the end of September 2018), which included 61 women and 39 men, and their ages ranged from 20 to 90 years old.

Genotyping

5 ml of peripheral blood was collected in tubes containing anticoagulant (EDTA-K_2). The tubes were transferred to the lab. DNA was extracted from a blood sample following a method previously reported [9]. Briefly, 5 ml of venous blood was added to a tube containing 100 ml of 0.5 M EDTA (as an anticoagulant) and mixed well so that it does not clot. Then, 2.5 ml of the sample was poured into the centrifuge tube and centrifuged for 10 minutes at 1500 rpm, and the serum was separated. With this process, we removed a series of proteins. In order to lyse the red blood cells, 5 ml of cold distilled water was added to about double the initial volume (2.5 ml), shaken well, and centrifuged for 30 minutes at 3500 rpm. At the end of the 3rd stage, we had a very compact white sediment at the bottom of the tube, and the supernatant solution contained red blood cells that had broken down. This solution removed by tilting the tube and shook it well and centrifuged it at 3500 rpm for 30 minutes. The rest of the blood cells were lysed, and the washing process was continued until the sediment was cleaned from the red blood cells.

Take out the supernatant solution again and put it into the tubes containing the sediment, which at this stage were about 0.25-0.5 cc, because the white blood cells swelled in the water, the amount of TES was 2.25 ml. We added it and shook well until the sediment was obtained, and it became soluble. We added 125

µl of 10% SDS. SDS is considered a cleaning agent that dissolves the fatty acids of the wall of white blood cells. Then, 50 µl of proteinase K with a concentration of 10 mg/ml was added, and after 3 to 24 hours, it was put in a water bath at 37°C. This step caused the rupture of the cell membrane and nucleus, and the DNA strands were released. After the desired time has passed and the white blood cells are lysed, 1.3 volume of the initial solution, which is usually 0.5-1. The saturated NaCl added to the tubes and shook it once or twice until it became cloudy and centrifuged for 30 minutes at 3500 rpm. The supernatant solution containing DNA was transferred to another tube, and isopropanol was added to 0.7 volume of the contents of the tube.

Then we transferred the DNA to a 1.5 ml microtube and centrifuged it at 8000 rpm for 30 seconds so that the DNA sticks to the bottom of the microtube, then emptied the supernatant containing isopropanol and salt, and then washed it twice with 70% alcohol. In order to wash the DNA, 1 ml of cold 70% alcohol (methanol) added to the microtube and after shaking the tube, centrifuged it for 30 seconds at 13000 rpm. Then the remaining alcohol removed and placed the tube containing DNA in the air for 30 minutes until the remaining alcohol evaporated. Then, 25 to 100 microliters of EDTA, TE (Tris-HCl) solution was added to the obtained DNA in proportion to its volume and put it in the refrigerator. PCR with a final volume of 25 µl was done that contain, 1µl of each reverse and forward primer (Sina gene) (table.1) 5 µl DNA,4 µl Master mix (Sina Gene),14 µl of distilled water. PCR conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 25 cycles at 94°C for 30 s, 61°C for 35 s, and 72 °C for 35 s, and a 4 min final extension at 72 °C.

Then 10 µl of PCR product mixed with 1.5 µl of the endonuclease enzyme AVAII (Sina gene) were placed in a bain-marie at 37 °C for 16 hours to investigate the digestion. PCR product of enzymatic digestion was investigated by 1 percentage of an agarose gel electrophoresis.

This study was approved by the ethical committee of Kerman University of Medical Sciences, and the Ethical approval Code is IR.KMU.REC.1399.286.

Statistical analysis

The data of genotypes of people with their age and sex in relation to consumption of warfarin were evaluated by software SPSS 20 ($P < 0.05$). The association between the daily dose of warfarin and *CYP2C9* polymorphism was examined via analysis of variance (ANOVA).

Table 1

*PCR primers and Restriction Enzyme used for PCR-RLFP OF the CYP2C9*2 gene*

Gene	Forward Primer	Rivers primer	Amplicon Size (bp)	Restriction enzyme
*CYP2C 9 *2*	5´- TGAGCTAACAACCA GGACTCAT-3´	5´- GGAGGATGGAAAAC AGAGACTTA-3´	395	AVAII

RESULTS

Patient characteristics

In this study, 100 patients were studied, and the maximum age of them was 90 years, and the minimum age was 20 years, with an average age and standard deviation 59/82±14/5. Also, the percentage of men and women was sequentially 39% and 61%. The subjects reviewed in this

study were divided into two categories (younger than 51, older than 51), in terms of the dose of warfarin usage. In these subjects, the average dose of usage of warfarin is concluded respectively 5/5296± 1/9385,4/0395 ±1/55869. Figure 1 shows the average daily dose of warfarin based on different polymorphisms of *CYP2C9*. Results of one-way ANOVA showed that there were no significant differences between different genotypes of *CYP2C9* polymorphism and an average daily dose of warfarin (P=0.999).

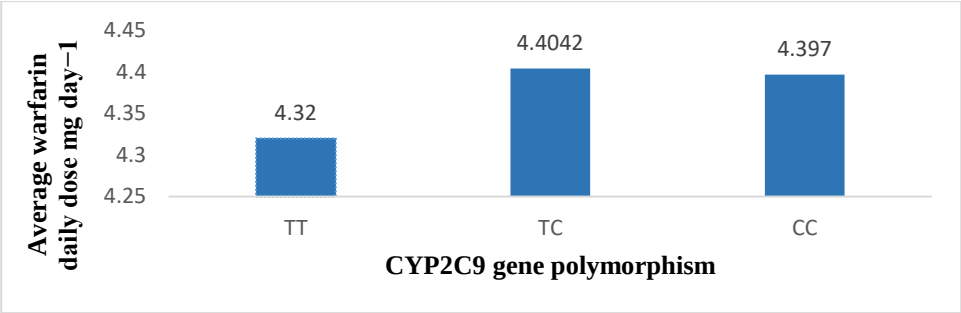

Fig. 1 Comparison of the average warfarin daily dose based on CYP2C9 gene polymorphism

The diagnostic distribution of patients was atrial fibrillation 55.4%, mechanical heart valve replacement 23.2%, and venous thromboembolism 21.4%.

Genotypic analysis

In order to identify the polymorphism of the *CYP2C9* gene, the PCR product (395 bp) was digested with the restriction enzyme AVAII, that in being of restriction site within the amplicon, digested it in two fragments of 170bp and 225bp, so in the samples.

With homozygous genotype TT because of enzyme indigestion, produce a pair of same fragments of 395bp and in patients with homozygous genotype CC because of enzyme digestion, produce fragment of 225bp, 170bp and heterozygote genotype TC produce three fragments (fig.2). The result of this enzyme digestion on the studied sample includes genotype TC=12(12%), CC=87(87%), TT=1(1%), So allele frequencies of T and C were 7% and 93%, respectively (fig.3).

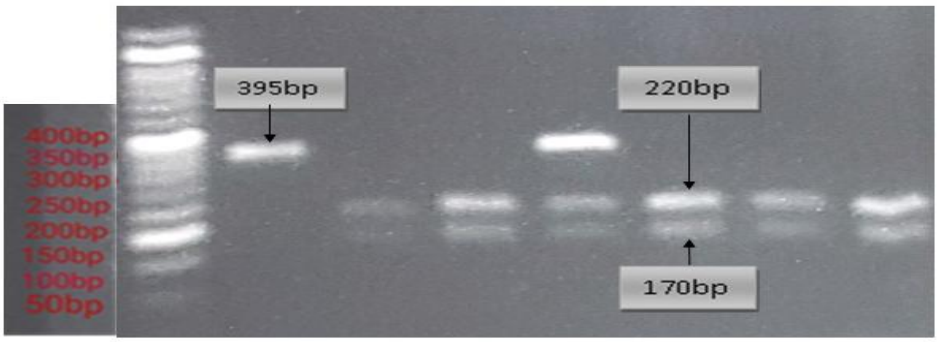

Fig. 2 Agarose gel electrophoresis showing PCR-RFLP products of the samples

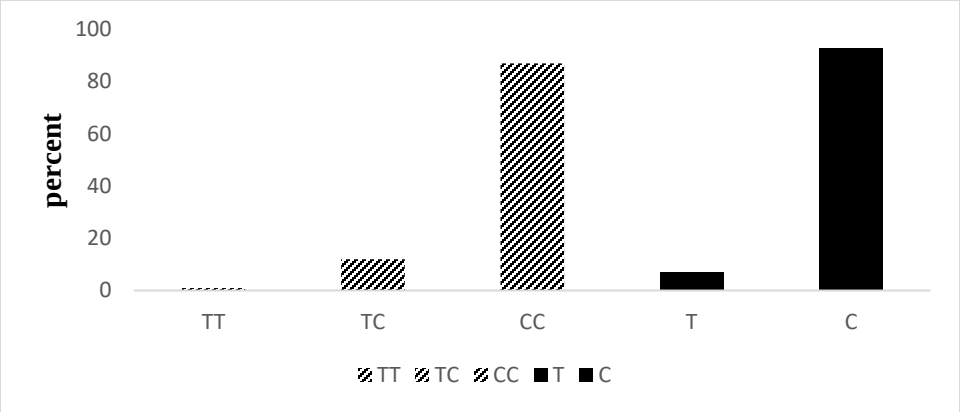

Fig. 3 Frequencies of CYP2C9 genotype and alleles in this study.

Based on the Table.2 There is meaningful relationship between age and the dose of warfarin usage (P=0.0001).

Table 2

Daily warfarin dose in 100 patients divided into two categories (younger than 51, older than 51)

Age of people under study	The average dose of warfarin mg/day	Number of persons	Standard deviation
Under 51years old	4/0395	76	**55869/1**
Over 51 years old	5/5296	24	**93855/1**
total	4/3971	100	**76707/1**

According to the information mentioned in the Table.3 It's clear that there is no meaningful difference between the two groups, women and men, in terms of genotype *CYP2C9* (P=0.115).

Table 3

The genotype distribution of the CYP2C9 polymorphism is associated with sex and age

genotype	female		male		Under 51 years old		Over 51 years old	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
TT	0	0/0%	1	2/6%	0	0/0%	1	1.3%
TC	5	8/2%	7	17/9%	4	16.7%	8	10.5%
CC	56	91/8%	31	79/5%	20	83.3%	67	88.2%
total	61	100%	39	100%	24	100%	76	100%

Based on the information in the above table, there is no meaningful relationship between the two age groups and the frequency of genotype polymorphism on the *CYP2C9* gene (P=0.626). Furthermore, there is no meaningful relationship between different genotypes of *CYP2C9* polymorphism and warfarin dose usage (P=0.999).

DISCUSSION

Adverse drug reactions is one of the serious health issues, which can be referred to admitting patients to the hospital, which has reported more than 100,000 deaths annually in the United States [9]. This type of variability in response to the drug is an important issue for a series of drugs with limited therapeutic indications. For example, warfarin has been recommended as an anticoagulant drug, which indicates high variability in drug dosage determination. Therefore, if it is not properly monitored, it will cause problems for patients; for example, inappropriate doses of warfarin may increase risks such as severe bleeding for patients. In general, identifying patients and being able to identify those who have a good or bad reaction before treatment can improve health care by increasing the beneficial effects and reducing the effects or side effects of the recommended drug [10].

Studies have shown that drug response is a complex phenotype or characteristic that depends on genetic factors and other factors such as age, health conditions, etc. But genetics plays an important role in drug response and variability. For example, CYP2C9 and VKORC1 polymorphisms play an important role in warfarin dose variability, which is independent of other non-genetic factors [11].

Warfarin is an oral anticoagulant and is used to treat and avoid thrombolytic disorders by inhibiting the vitamin K-dependent coagulation pathway. Despite its effectiveness, this substance has a therapeutic index, and the reaction to it is completely variable. A large dose of this drug leads to bleeding, and a low dose leads to undesirable blood coagulation. Many attempts have been made to determine the dose of warfarin based on age, sex, diet, etc [12]. Cardiovascular diseases are among the first three causes of mortality and disability in the world. In the past, infections and contagious diseases have been a cause of death, but nowadays, non-communicable diseases have become more dangerous [13]. These damages can be due to genetic factors and acquired changes involved in the blood coagulation system, in addition to known environmental and acquired factors. Polymorphism of two genes, CYP2C9 and VKORC1, has been identified as a genetic factor involved in the dose required of warfarin in these patients [14]. Enzyme *CYP2C9* and its common polymorphisms are effective in the amount of expression and as a result of protein products, so changing mutations in the gene lead to changes in expression that can determine the activity of this enzyme in the metabolism of vitamin K. Several algorithms have been developed, with the aim of reducing the risk of bleeding, personalized treatment, reduces the cost and hospitalization time and in fact, to determine the dose of warfarin to enhance the performance and safety of treatment with warfarin. Some items in these variables algorithms are: weight, age, gender, and genotype *CYP2C9*, *VKORC1* [15, 16]. For patients with genetic variation, the dose of warfarin with polymorphism in *CYP2C9* and *VKORC1* genes should be lowered at the beginning of treatment, and the conditions of these people should be handled closely to stabilize the dose [17]. In this study, the genotype polymorphism of the CYP2C9 gene was compared between women and men. The results showed that there is no difference between men and women in the genotype polymorphism of the *CYP2C9* gene (P=0.115). Another study that was conducted by Tabatabai et al [18] in 2011 using the technique RFLP-PCR for 200 heart patients on the *CYP2C9* gene in Tabriz showed that warfarin had a significant

relationship with gender and was more common in males than females. In the present study, there was a meaningful relationship between the dose of warfarin usage and the age of people (P=0.0001), but in other studies that was conducted by Fariba Rad et al [19] and Popak et al [20], there was no meaningful relationship between age and the dose of usage of warfarin usage. Furthermore, in this research affected people divided into two age groups (51 years, older than 51 years, younger than 51 years), result showed that there is no meaningful difference between the frequency of polymorphism and these two age groups, but in another study that conduct by tataronase et al in 2011 the daily dose of warfarin has showed meaningful relationship with weigh, height and BMI of patients [21]. In this study the dose of usage of warfarin surveyed in terms of the genotype polymorphism of *CYP2C9* gene and result showed that there is no meaningful difference between the dose of usage of warfarin and *CYP2C9* gene polymorphism, as well in another study that conducted in the population of Indian and singaporeans no relationship was found between the dose of usage of warfarin and polymorphism of *CYP2C9* gene [22]. An inspection by Fariba Rad et al showed that the polymorphism of gene CYP2C9 plays an important role in the dose of warfarin usage [19].

CYP2C9 is a third gene affected by the dose of warfarin [23]. An reserch accompolished by behzad popack et al [20] in 2014 and 2015 it is concluded that there is meaningful relationship between polymorphism *VKORC1*and *CYP2C9* genes and the dose of usage of warfarin. Kianmehr et al [24] in 2008 showed that the sensitivity of warfarin is a multi-agent and multigene phenomenon. But the combination of genetic polymorphisms has the most effect on the sensitivity of warfarin. There is a meaningful relationship between variants of the gene VKORC1 and the CYP2C9 genes with the dose of warfarin usage. Studies conducted by Popak et al [20], Reyhane et al [25], and Mazzaccara et al [26] showed that there is a meaningful relationship between the genotype of polymorphism CYP2C9 and the dose of warfarin usage, which is dissimilar to this study.

CONCLUSION

The results of some previous studies show that there is a meaningful relationship between the genotype of the polymorphism of the CYP2C9 gene and the dose of warfarin usage in patients. In this study, it seems that the dose of warfarin usage in patients is a multifactorial phenomenon that can be affected by different genes, dietary, and environmental factors that should be studied.

Transparency declaration

There is no conflict of interests.

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