Cytochrome-P450 2C9 Polymorphisms: Contribution to Warfarin Sensitivity and Prevalence in Egyptian Population

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ABSTRACT

Cytochrome P450 2C9 is the main enzyme for degradation of many drugs including the most widely prescribed oral anticoagulant; warfarin. It is polymorphic with three common allelic variants: The wild type CYP2C9*1(the most active), and CYP2C9*2 and CYP2C9*3 both have less catalytic activity . The distribution of CYP2C9 variants varies greatly from one population to another. The aim of the present study was to assess the contribution of CYP2C9 polymorphims in the dose requirement of warfarin in Egyptian population and to estimate the prevalence of different CYP2C9 alleles and genotypes in the Egyptian population. One hundred and ninety six patients on warfarin therapy (INR target between 2.0 and 3.0) and 150 normal subjects were included in the present study. The genotypes were determined using PCR-RFLP.
Results: *Allele frequencies in the Egyptian population sample were 70%, 20.1% and 9.9% for CYP2C9*1, CYP2C9*2 and CYP2C9*3 respectively. Cases carrying either CYP2C9*2 or CYP2C9*3 required lower doses of warfarin for proper therapeutic response than those with the wild allele.* ***Conclusion****: There is high frequency of CYP2C9*2 and CYP2C9*3 variants amoung Egyptian population. These two variants are related to lower warfarin dose requirements which may lead to over anticoagulation and unstable anticoagulant response.*
Key words: *Cytochrome P450 – polymorphism - oral anticoagulation - pharmacogenetics.*

INTRODUCTION

Pharmacogenetics is the special area of biochemical genetics that deals with the variability in response to drugs that is due to genetic variation. That genetic variability might alter the ability of the body to absorb, transport, metabolize and excrete drugs or their metabolites and so it is partially responsible for the divergence in both drug response and adverse drug reaction. Identifying the genetic loci reponsible for such variations might enable individually-designed drugs with maximum drug efficacy and mininal side effects[1].

Warfarin is the most widely prescribed oral anticoagulant to prevent recurrent arterial and venous thrombosis. However, optimal use of the drug has been hindered by its

narrow therapeutic index (the range of doses between the minimal dose required for therapeutic effect and the maximum dose without manifesting adverse reactions). In addition, warfarin has a great interindividual variability in the doses needed to achieve optimal therapeutic response[2]. Bleeding, the most important side effect of warfarin, is relatively common. It has been estimated that 7.6 to 16.5/ 100 patients on warfarin per year manifest haemorrhage which is life threatening in about one sixth of them[3,4]. Warfarin is produced as a racemic mixture of R-warfarin and S-warfarin, but the predominance of pharmacologic activity resides in the S-enantiomer[5]. S-warfarin is mainly metabolized by cytochrome P450 CYP2C9[6].

The cytochromes P450 (CYP) are a group of related enzymes that are found in nearly all animal species. Among more than 20 different CYP enzymes, only six (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A) account for the metabolism of nearly all clinically useful medications[7]. Although the enzymes are somewhat related and share some general characteristics, each one is unique and has a distinct role. CYP2C9, a cytochrome P450 enzyme belonging to family 2 and subfamily 2C, is responsible for the metabolism of several common medications including many of the nonsteroidal anti-inflammatory drugs (NSAIDs), phenytoin, tolbutamide, glipizide, celecoxib, fluvastatin and (S)-warfarin[8].

The CYP2C9 has been demonstrated to be polymorphic, and its genetic variability has been shown to be associated with variations in the levels of enzyme activity and hence for the level of the active drug. In addition to the wild-type CYP2C9*1 (which has the highest catalytic activity), there are two polymorphic loci CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu) have been identified and showed significant effect on the turnover of drugs metabolized by that enzyme e.g warfarin. Six different genotypes are produced by combination of these alleles; 1*1*, 1*2*, 1*3*, 2*2*, 2*3* and 3*3*[9] .The distribution of these polymorphic alleles varies widely from population to another e.g. while 23 percent of Caucasians have one or more altered CYP2C9 genes, these polymorphisms are present in only 2% of African Americans[10].

The aim of the present work was to investigate the effect of CYP2C9 variants in the dose requirements of warfarin in Egyptian patients and to determine the frequencies of CYP2C9 variants among Egyptians.

SUBJECTS & METHODS

A total number of 346 subjects were included in the study. They were included into 3 groups; Cairo group, Alexandria group and normal control group. Cairo group cases (70 cases) were recruited from the Department of Vascular Surgery, Cairo University Hospitals. Alexandria group (126 cases) was selected from the Outpatients' Clinic of the Vascular Surgery Department, Alexandria University Hospitals.

All subjects from both groups (196 cases) were deep venous

thrombosis (DVT) cases on oral warfarin treatment for at least five days either in the acute phase of the disease or taking the medication for prevention of the recurrence and were within the therapeutic range (International Normalized Ratio [INR] between 2 and 3). Cases with liver diseases, heart diseases, or who are heavy smokers (known conditions that affect the dose of the oral anticoagulant), or those whose INR were outside the therapeutic range were excluded from this study.

Human Genetics Department, Medical Research Institute, Alexandria University is a referral center for genetic disorders and for premarital counseling. One hundred and fifty unrelated subjects selected randomly from the DNA samples present at the Human genetics Department, Medical Research Institute, Alexandria University were also included in the study. These samples were taken from normal individuals attended to the Clinics for premarital counseling or carrier detection for any other genetic disorders provided by the Molecular Genetics Laboratory, Medical Research Institute, Alexandria University. All included control subjects gave informed consent to be included in the study. Forty four of them were originally from Alexandria, 29 from El-Behera governorate, 12 from Matrouh, 43 either from or their parents from Upper Egypt, 19 from the Delta, and 3 were from Cairo.

All cases from both groups (Cairo and Alexandria) were subjected to the following:

I. **Thorough history taking** including: medical history.

II. **Examination** including: general examination and vascular examination.

III. **Measurement of the prothrombin time converted into INR value.**

IV. **Molecular Genetic testing for both cases and control**

1. **Genomic DNA was extracted** from peripheral EDTA treated blood cells by DNA salting out as described by *Sambrook et al* [11].

2. **Detection of CYP2C9 (Arg144Cys) gene polymorphism using PCR-RFLP** (The allele coding for Cysteine abolishes a restriction site for the *Eco*47I (*Ava*II) that removes 57 bases from 423 base pairs of the PCR product)

a. **PCR** was carried out in a thin 0.2 ml tube. The reaction mix when brought to the final volume of 50 μl contains ≈ 300ng DNA, 3mmol/L $MgCl_2$, 0.4 mmol/L each of the dNTPs, 1X reaction buffer (75mM Tris-HCl; pH 8.8), 20mM $(NH_4)_2SO_4$ 0.01% Tween 20), 0.3μmol/L of the two oligonucleotide primers (forward and reverse), and 1.25 U of *Taq* DNA polymerase (MBI Fermentas). The primers were those described by *Tassies et al* [12].

Cycling program using the ThermoHybaid PCR Express Thermal Cycler was as follows: initial denaturation at 95° for 5 min, followed by 37 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 45 sec, elongation at 72°C for 45 sec, and a final elongation step at 72°C for 5 min.

A fragment of 423-base pair was obtained after electrophoresis of 10 μl

of the PCR products on 2% agarose gel stained with ethidium bromide.

b. **Restriction enzyme digestion** was carried out in 40-µl reaction volume containing 20 µl precipitated PCR product, 4 µl 10 x R+ Fermentas buffer (10mM Tris-HCl; pH 8.5), 10mM MgCl2, 100mM KCl, and 0.1mg/ ml BSA), and 10 units of the restriction enzyme Eco47I (MBI Fermentas). The reactions were incubated at 37°C for 16 h before electrophoresis.

Electrophoresis was carried out on a 3.5% agarose gel and visualized after staining with ethidium bromide. The wild allele (codes for Arginine amino acid) shows 2 fragments of 363-bp and 57-bp (usually run away) while the other allele that codes for Cysteine amino acid shows only one fragment at 420-bp.

1. **Detection of CYP2C9 (Ile359Leu).** A mismatched PCR primer was used that creates an *Mph*1130I restriction site when the allele codes for Leucine amino acid is present, the primers are listed in *Tassies et al* [12] PCR amplification and restriction digestion was done using a similar protocol like that mentioned for Arg144Cys but differs in the annealing temperature (59 °C).

The digested product was analysed in a 3.5 % agarose gel electrophoresis. The wild allele (codes for Isoleucine amino acid) shows fragments of 131-bp while that codes for Leucine amino acid shows fragments at 110-bp and 21-bp (usually run away).

V-Statistical Analysis

Statistical differences between allele and genotype frequencies were determined with the Chi-square statistic with significance set at <0.05. with 95% confidence intervals computed with the logit method.

Expected genotype frequencies were calculated using the Hardy–Weinberg equation ($p2 + 2pq + q2 = 1$). The chi-squared test was used to compare allele frequencies in the three groups and in the total sample. P-value of 0.05 or less was regarded as significant.

RESULTS

The frequencies of the CYP2C9*2, CYP2C9*3, variants in 196 cases treated with warfarin (70 from Cairo University Hospital and 126 from Alexandria University Hospital) and in 150 general population controls were analyzed using PCR-RFLP (Figures 1 - 4). Table 1 shows that 61.4% of Cairo cases, 56.3% of Alexandrian cases, and 50% of the controls were males.

Table 1: Sex of the different groups

	Cases						Control	
	Cairo cases		Alex Cases		Total			
	n	%	n	%	n	%	n	%
Male	43	61.4	71	56.3	114	58.2	75	50
Female	27	38.6	55	43.7	82	41.8	75	50
Total	70	100	126	100	196	100	150	100

The minimum, maximum, average and standard deviation of the age, INR and the maintenance dose of warfarin in cases are shown in table 2. The mean warfarin dose ±SD in Cairo group was 8.1 ± 3.3, mg while it was 7.6 ± 3.4 mg in Alexandria group. The mean INR were 2.4 ± 0.3 in both groups. The mean maintenance dose of warfarin was higher in the wild allele than in the other two alleles. There is no statistically-significant correlation between INR and the age, INR and the warfarin dose or between age and the dose of warfarin in both groups.

Table 2: Age, INR and maintenance dose of warfarin in different groups included in the study.

	Cairo Cases (n=70)				Alex Cases (n= 126)				Total cases			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Age(years)	20	70	47.9	11.2	24	78	53.4	12.2	20	78	51.4	12.1
INR	2	3	2.4	0.3	2	3	2.39	0.3	2	3	2.4	0.3
Dose (mg/day)	1.5	15	8.1	3.3	1.5	15	7.6	3.4	1.5	15	7.8	3.4

INR: International normalized ratio; commonly adopted for laboratory monitoring of oral anticoagulant therapy and as a guide to warfarin dose adjustment [13].

The frequency of CYP2C9*1 allele (wild allele) among Alexandria cases (table 3, figure 5), Cairo cases and the control groups genotyped person was 70%, and at least one mutant allele were present in 30% of the sample population. The least common allele was CYP2C9*3 with frequency of 12.6, 9.2 and 9.3 % in Cairo cases, Alexandria cases and control group respectively. Allele CYP2C9*2 variant was

present in 18.6% in both groups of cases and 22% in the control group with average

of 20.1% in the total sample. There was no statistical significant difference between all groups regarding the frequency of different alleles.

Table 3: Alleles and genotype distribution among groups distributed in the study

	N	Alleles (%)			Genotype (%)					
		1*	2*	3*	1*1*	1*2*	1*3*	2*2*	2*3*	3*3*
Cairo Cases	70	68.8	18.6	12.6	48.6	22.9	17.1	5.7	2.8	2.9
Alex Cases	126	72.2	18.6	9.2	54.8	24.6	10.3	6.3	0	4
Total Cases	196	71	18.6	10.4	52.6	24	12.7	6.1	1	3.6
Control	150	68.7	22	9.3	50.7	23.3	12.7	9.3	2	2
Total sample	346	70	20.1	9.9	51.8	23.7	12.7	7.5	1.4	2.9

Genotypes frequencies of different alleles are shown in table 3 and in figure 6. The most common genotype was the homozygous for the wild allele followed by heterozygous of wild allele (1*2* and 1*3*) in all groups. They constitute 88.6 %, 89.7% and 86.7% in Cairo cases, Alexandria cases and control groups respectively. These genotypes were represented in 88.2% of the total sample population. The least frequent genotype was the heterozygous of both variants (2*3*) in all groups which was not detected in the Alexandria cases group. Homozygous for the variants (2*2* and 3*3*) were detected in all groups. No statistically-significant correlation was detected between genotypes and INR

The frequency of different alleles and the distribution of genotypes do not differ significantly from one group to the other. Alleles and genotype proportions were in Hardy-Weinberg equilibrium in cases (Alexandria and Cairo groups), control and in the total sample population.

The maintenance dose of warfarin was significantly higher in allele one relative to the other 2 alleles (table 4). Such finding was statistically of higher significance in Cairo cases and in combined groups (Alexandria cases and Cairo cases) $P < 0.001$ and 0.006 respectively. No statistically-significant correlation between different alleles and INR.

Table 4: Mean doses of Warfarin in relation to CYP2C9 genotype in 196 patients with a target INR (2.0-3.0)

Genotype	1*1*		1*2*		1*3*		2*2*		2*3*		3*3*	
	no.	Mean dosage (mg/day)	no.	Mean dosage (mg/day)	no	Mean dosage (mg/day)	no	Mean dosage (mg/day)	no	Mean dosage (mg/day)	no	Mean dosage (mg/day)
Cairo cases	34	9.7	16	7	12	5.6	4	6.8	2	8.7	2	7.5
Alexandria cases	69	9.2	31	7.2	13	6.4	8	6.1	0	0	5	6.8
Total cases	103	9.4	47	7.1	25	6	12	6.3	2	8.7	7	7

Figure 1: PCR products of an area spanning CYP2C9 Arg144Cys polymorphic site. M is a 50bp Molecular weight DNA marker. The PCR products is 420 bp.
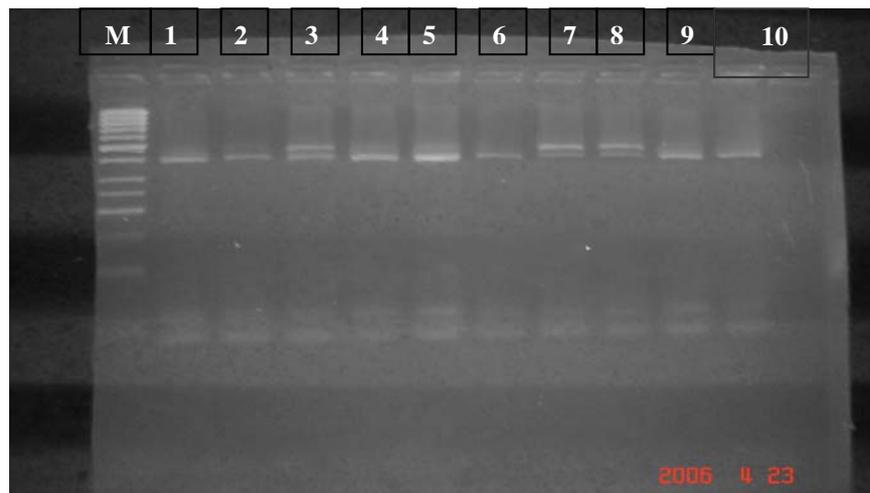
Figure 2: Restriction digestion for PCR products of the area spanning CYP2C9 polymorphic site at amino acid 144 on 3.5% agarose gel stained with ethidium bromide. M is 50bp molecular weight DNA marker, lanes (1, 2, 4, 5, 6, 9 and 10) are homozyzous for Arg. Lanes (3, 7 and 8) are heterozygous for Arg and Cys at that site.
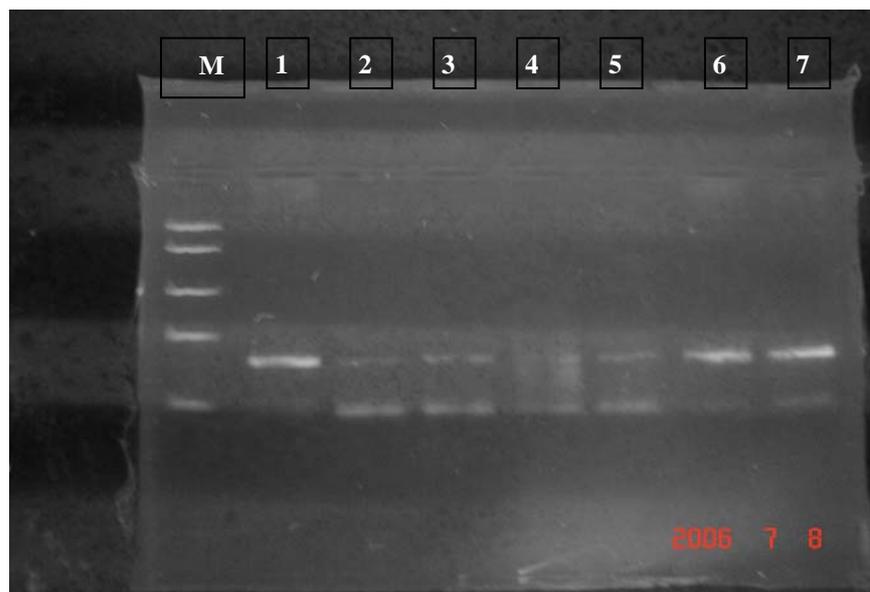

Figure 3: PCR products of an area spanning CYP2C9 Ile359Leu polymorphic site on 2% agarose gel stained with ethidium bromide. M is a Fast Ruler Molecular weight DNA marker (Fermentas MBI). The PCR products is 131 bp.
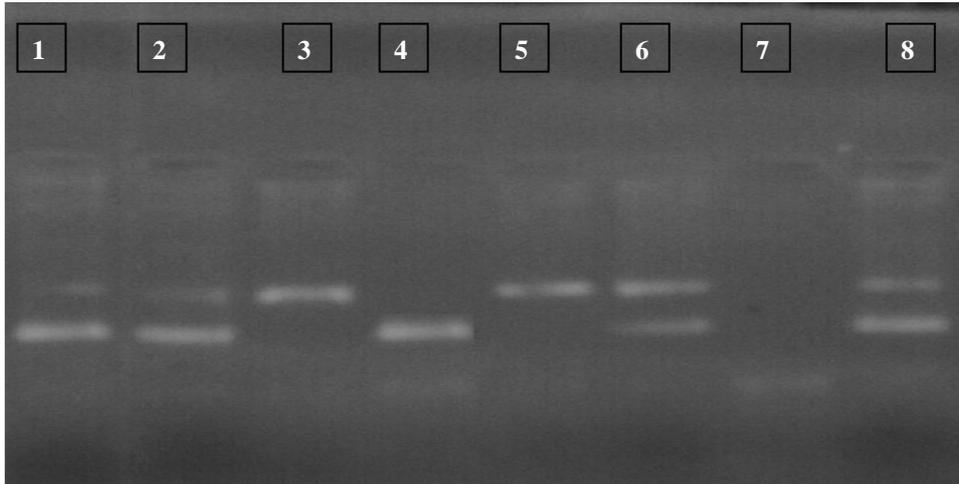
Figure 4: Restriction digestion for PCR products of the area spanning CYP2C9 polymorphic site at amino acid 359 on 3.5% agarose gel stained with ethidium bromide. Lanes (3 and 5) are homozygous for Ile. Lane 4 is homozygous to Leu. Lanes (1,2,6 and 8) are heterozygous.
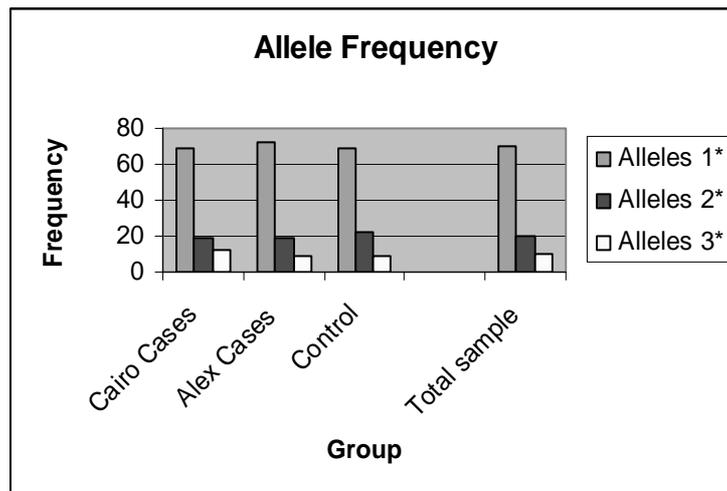

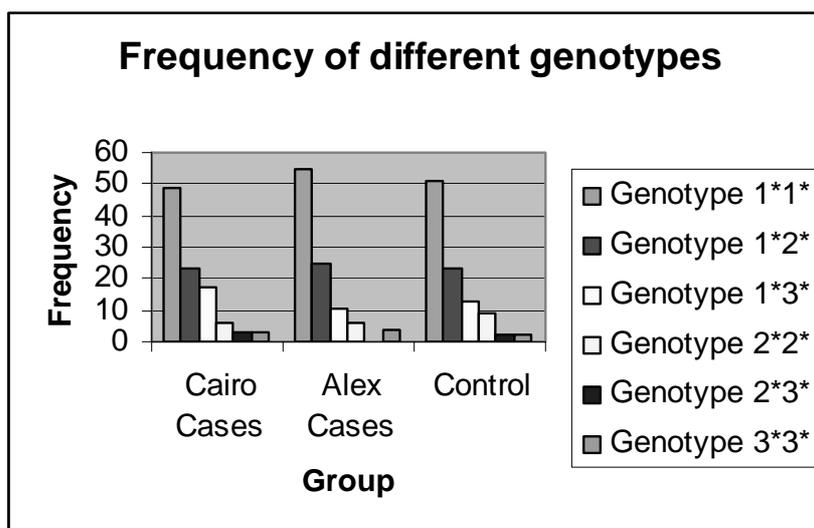*Figure 5: Allele frequency among different groups included in the study.*

Figure 6: Genotypes distribution among groups included in the study.

DISCUSSION

Warfarin therapy is complicated by large interpatient variability in maintenance dose requirement and the associated risk of under- and over-anticoagulation[14]. Genetic factors determining the activity of CYP2C9 have been demonstrated to be important. Two variants, Arg144Cys (CYP2C9*2) and Ile359Leu (CYP2C9*3), have reduced enzyme activity compared to the wild type (CYP2C9*1)[15].

Genetic factors are not the only factors that can affect the maintenance dose of warfarin. Some medical conditions may also affect it. Hepatic and renal diseases may affect the required doses by changing the drug absorption, metabolism, and excretion or even the synthesis of the clotting factors. Cases with cardiac lesions and those who are heavy smokers, through relative hypercoagulable state, may need higher doses for warfarin to reach the therapeutic index [16]. In the present study, any case with detectable hepatic or cardiac abnormalities or heavy smoker was excluded.

Only cases with INR between 2 and 3 were included in the current study. That may be the explanation why there were no correlation between INR and any allele as detected in some other studies [17,18,19]. Also, there was no correlation between the age and dose of warfarin in the present study because it is a minor factor that needs larger sample size to be detected.

In the present study, the maintenance dose of warfarin in

relation to the common CYP2C9 variants was tested. Patients with CYP2C9*2 and CYP2C9*3 required lower doses of warfarin for proper therapeutic effect. These results are consistent with other reports suggesting that decreased catalytic activity of both isoforms CYP2C9*2 and CYP2C9*3 results in long half life of the drug. This decreases the maintenance doses for the targeted therapeutic effect[9,17,18,19,20].

Several studies reported that the CYP2C9*3 variant has greatly reduced the catalytic activity even more than CYP2C9*2[21,22,23]. However the current study, could not detect a significant difference in the maintenance dose of warfarin in cases with CYP2C9*2 allele in relation to cases carrying CYP2C9*3 allele. Moreover, although the present study reported homozygous of CYP2C9*3 in 3.6% of cases, no statistically-significant lower doses in that group relative to those with homo or heterozygous of CYP2C9*2 variant was evident. The relatively small number of cases with CYP2C9*2 or CYP2C9*3 variants might limit the ability to detect such possible correlation especially if the difference in the catalytic activities between both alleles is not so evident.

The distribution of CYP2C9 variants varies greatly from one population to another. Table 5 shows the distribution of these alleles among different populations. The prevalence of CYP2C9*2 and CYP2C9*3 variants are more common in Caucasians especially around Mediterranean Sea. The alleles' frequencies in the present study are comparable to those in Spain [24]. The main difference is the relatively higher prevalence of CYP2C9*2 variant in the current study population (20.1%) relative to the study done on Spanish (15.6%). The frequency of CYP2C9*3 in Spain (9.8%) is almost the same as in the present Egyptian sample (9.9%).

Table 5: The frequency of CYP2C9 polymorphic alleles in different populations

Population	n	Allele			Reference
		*1	*2	*3	
Egyptian	346	70	20.1	9.9	*Present study*
Egyptian	274	82	12	6	*25*
White American	100	96	8	6	*10*
African Americans	100	89.5	1	0.5	*10*
Swedish	430	81.9	10.7	7.4	*26*
British	100	79	12.5	8.5	*27*
Japanese	218	97.9	0	2.1	*28*
Chinese-Taiwanese	98	97.4	0	2.6	*10*
Korean	574	98·9	0	1·1	*29*
Italian	157	79·6	1·12	9·2	*30*
Ethiopian	150	93·33	4·3	2·3	*30*
Tamilian	135	90·7	2·6	6·7	*31*
Beninese	111	95·5	0	0	*32*
Belgian	121	82·2	1·0	7·4	*32*
Russian	290	82·6	10·5	6·7	*33*
Turkish	499	79·4	10·6	1·0	*34*
Spanish	102	74·5	15·6	9·8	*24*
Malays	202	95·7	1·9	2·4	*35*
Chinese	165	97·3	0	3·3	*35*
Indians	165	88·2	2·1	9·7	*35*

Hamdy et al 2002[25] reported that the frequencies of CYP2C9*1, CYP2C9*2 and CYP2C9*3 variants were 82, 12 and 6% respectively in the Egyptian population. Their results are away from the present study results. However, the difference in the sample populations might have the clue for such a difference. In the study of Hamdy et al[25], all subjects were either students or staff members in Cairo University. The authors hypothesized that students and professors of Cairo University represent the Egyptian population [25]. The present study sample was cases and control from Cairo, Alexandria, Delta, Matrouh and Upper Egypt. Furthermore, while in the previous study, the researchers refer that Bedouins and Nubians were represented in their samples, none of our subjects was related to these minority groups in Egypt.

Although we think that neither our sample nor the sample of the previous study[25] could represent the Egyptian population precisely, we presume that our sample is more representative for the Egyptian population than that of the previous study. It is important to highlight their hypothesis of the movement of many Egyptians from Upper Egypt and Delta to Cairo.

During its long history, Egypt accepted a lot of immigration waves and interacted with different populations. Egypt was a part of many empires (Egyptian, Greek, Roman, Arab, Ottman, French, and British). Some of these empires extended as far as Somalia in the South, to Morocco in the west, to the China borders in the East and to the center of Europe in the North. This allowed interaction between Egyptian population and many different ethnic groups. Although we think that such history must be imprinted somehow in the genetic pool of the modern Egyptians, there are no enough studies reconstructing that history from the genetic point of view. For CYP2C9 variants, there is no enough data about the frequencies of these variants in other populations around Egypt.

The most accepted explanation for the wide variation in CYP2C9 gene and in other genes encoding enzymes important for detoxification of drugs and xenobiotics in general is that it is an evolutionary adaptation to exposure to some toxins during the history of certain ethnic groups [36].

Cumarines including warfarin are common in many plants and has been used as a medicine for hundreds of years. They have some beneficial effects as a treatment of intestinal parasites, brain stimulants or depressants, insect repellents, mold growth inhibitors..….etc. On the other hand, they have some deleterious effects as they are highly carcinogenic. They are present in many plants that have been consumed by the human kind e.g. wild potatoes, sumac berries, juniper berries, purslane leaves, sage, chickpeas, coriander, parsnips, plums, oranges and fig [37]. For an evolutionary theory to be accepted, long history of exposure is mandatory [36]. According to that theory, relatively high frequency of CYP2C9 variants is the result of long history of exposure to cumarines by either Egyptians or other ethnic groups that amalgamated into the Egyptian gene pool by

immigration. Although many of the forementioned plants are common in the modern Egyptian cuisine, we did not find data about when these plants were introduced in Egypt.

In the present study, the alleles and genotypes frequencies are in Hardy-Weinberg equilibrium. Such finding may support the long term exposure adaptation theory and not the immigration as the source of the high frequency of CYP2C9 variants[36]. As expected for the frequency of certain allele due to immigration, it will not be in Hardy-Weinberg equilibrium. However, in a community like Egyptian with incidence of consanguineous marriage (approximately 28%)[38], it is also unexpected to find alleles and genotypes in Hardy-Weinberg equilibrium. So, we can not outweigh any of these possibilities based on Hardy-Weinberg equilibrium.

In conclusion, there is a high frequency of CYP2C9*2 and CYP2C9*3 variants among Egyptian population. That high frequency could be the result of an evolutionary adaptation to consumption of food rich in cumarines for centuries. It could be, also, due to immigration from different ethnic groups with high prevalence of these variants. These variants affect the metabolism of some important drugs and may increase the risk of side effects due to their decreased catalytic activity resulting in long half life of the drug.

ACKNOWLEDGMENT

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التعدد الشكلي لإنزيم سيتوكروم بي 450 2ج9 : إسهامه في ضبط الجرعة لعقار "وارفارين" ، وانتشاره في الشعب المصري

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سيتوكروم بي 450 2ج (سي ب 2 ج 9) هو الانزيم الأساسي في تكسير عدة عقارات مثل عقار وارفارين الذي يستخدم بكثرة لمنع تجلط الدم .

والتعدد الشكلي لهذا الإنزيم له ثلاثة بدائل : (سي ب 2 ج 1 9 [*]) وهو الأكثر نشاطا ، (سي ب 2 ج 2 9 [*]) و(سي ب 2 ج 9 3 [*]) وهما الأقل نشاطا.

كما أن توزيع هذه البدائل يختلف من شعب لآخر.

ويهدف هذا البحث إلى توضيح دور التعدد الشكلي لهذا الإنزيم في تحديد جرعة عقار "وارفارين"، كما يهدف إلى تحديد انتشار بدائل هذا الإنزيم في الشعب المصري .

وقد أجريت الدراسة على 196 مريض يستخدم عقار "وارفارين" و 150 شخص طبيعي. وقد استخدم تفاعل البلمرة المتسلسل وإنزيمات قطع الحامض النووي لدراسة التعدد الشكلي للإنزيم وتحديد البدائل .

وقد وجد أن نسبة وجود بدائل هذا الإنزيم في هذه العينة من الشعب المصري هي كالتالي :

70 % للبديل الأول 1 [*] وهو الأكثر نشاطا، 20.1 % البديل الثاني 2 [*] و 9.9 % البديل الثالث 3 [*].

كما وجد أن حاملي البدائل 2 [*] و 3 [*] يقل احتياجهم لجرعة الوارفارين للوصول للنتائج المرجوه للعلاج ، مما قد يؤدي إلى سيولة دم هؤلاء الأشخاص بنسبة أكبرعند استخدامهم لجرعة قليلة من العقار مقارنة بحاملي البديل 1 [*].