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Genetic contribution of *CYP2C9*, *CYP2C19*, and *APOE* variants in acenocoumarol response

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ABSTRACT. Oral anticoagulants of the coumarin type have an inconveniently narrow therapeutic window, making their use difficult. In Mexico, genetic variables that participate in the heterogeneity of the therapeutic response remain poorly investigated. With the focus on warfarin, extensive pharmacogenomic studies have been performed, including those on the CYP450 family and *APOE*. The objective of this study was to determine the contribution of *CYP2C9*, *CYP2C19*, and *APOE* polymorphisms to the variations in response to the doses of acenocoumarol, which is the main

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anticoagulant prescribed to the Mexican population. The polymerase chain reaction–restriction fragment length polymorphism method was applied to identify *2 and *3 of *CYP2C9*, *2 of *CYP2C19*, and *APOE* variants. The genetic distribution of every polymorphism tested showed high variability when compared with other populations worldwide. Our results showed statistical differences only in the *CYP2C19* gene between the *1*1 and *1*2 groups, with effective acenocoumarol doses of 2.56 ± 1.34 mg/day *vs* 1.35 ± 0.84 mg/day (P = 0.005), respectively. Multiple regression analysis, including patient age and both the *CYP2C9* and *CYP2C19* genes, showed that these variables explained more than 20% of the dose variations. This is the first report in Mexico searching for the relationship between CYP450 and *APOE* polymorphisms and the dose requirements of acenocoumarol. Our results suggest that, in the Mexican population, *CYP2C19* is more involved in acenocoumarol metabolism than *CYP2C9* and *APOE*. Besides considering the age factor, pharmacogenetic testing for *CYP2C19*2* before initiating acenocoumarol treatment could lead to a safer anticoagulation therapy in Mexican patients.

Key words: *CYP2C9*; *CYP2C19*; *APOE*; Genetic polymorphisms; PCR-RFLP; Acenocoumarol

INTRODUCTION

Vitamin K is involved in the carboxylation of glutamate motifs to form gamma-carboxyglutamate residues. Such proteins, prone to carboxylation, are coagulation factors (II, VII, IX, and X) anticoagulant proteins (protein C and S), proteins involved in bone metabolism (osteocalcin, periostin) and vascular biology (growth arrest-specific protein 6) (Coutu et al., 2008). Otherwise, through apolipoprotein E (Apo E), dietary vitamin K is transported to the liver, where is converted to the reduced form in order to exert its coagulant activities (Kohnke et al., 2005).

Oral anticoagulants (OAs), mainly warfarin and acenocoumarol, have been used to prevent and treat cardiovascular diseases. These coumarin derivatives inhibit the enzymatic conversion of inactive vitamin K epoxide to its reduced active form, preventing blood coagulation (Anonymous, 1998).

The international sensitivity index (ISI) establishes the OA doses that take into consideration the disease to treat, individual response, and drug adverse effects. Default ISI values range from 2 to 3.5; however, some patients (drug resistance) need higher doses of OA to reach the required ISI, and others present adverse reactions (hypersensitivity) at recommended doses (Anonymous, 1998).

The plasma clearance variability of OAs is, in part, genetically determined by several enzymes of the cytochrome P450 family, which catalyze the oxidative metabolism of OAs. CYP2C9 and CYP2C19 are the major enzymes responsible for the hepatic metabolism of OAs as well as other common drugs are used in the clinical area, including many proton pump inhibitors and antiepileptics. These enzymes hydroxylate the *S*-enantiomers of warfarin, acenocoumarol, and phenprocoumon (Daly and King, 2003). *CYP2C9*2*, *CYP2C*3*, and

*CYP2C19*2* are the main polymorphisms studied and have been related with OA dose variations (Lee et al., 2007).

APOE is a glycoprotein encoded by the human *APOE* gene. There are three isoforms encoded by 3 *APOE* alleles; namely, e2, e3, and e4. It has been proposed that APOE polymorphisms might influence the uptake of vitamin K into hepatocytes and OA efficacy (Kohnke et al., 2005; Wadelius et al., 2007).

The reported pharmacogenomic data refer mainly to warfarin treatment, since it is commonly prescribed as an OA. However, a high rate of side effects and a low therapeutic window have been associated with this drug, necessitating the consideration of alternative pharmaceutical formulas (Hamby et al., 2000). In Mexico, the genetic variability related to OA dosage has been poorly investigated. The objective of this study was to determine the genetic contribution of polymorphisms in CYP and APOE to the acenocoumarol dose variations in Mexican patients. We studied the functional polymorphisms of *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *CYP2C19*2* (rs4244285), and *APOE* *2, *3, and *4 (rs429358/rs7412) in patients treated with acenocoumarol.

MATERIAL AND METHODS

Study universe

A total of 71 patients receiving acenocoumarol were enrolled from the cardiology, hematology, and medicine departments of Centro Medico Nacional de Occidente, IMSS in Guadalajara, Mexico. Only patients living in Western Mexico and whose daily acenocoumarol dose had been stable for at least 1 month were included. Those who presented side effects, and for whom treatment needed to be suspended, were also considered. Patients with compromised liver function (aminotransferase 1.5 times higher than normal values) or renal function (creatinine values higher than 1.5 mg/dL), and those on medications known to interact with coumarinic metabolism, were excluded from the study. All patients gave written informed consent to participate in the study.

Genotyping

*CYP2C9*2* and **3*, *CYP2C19*2*, and *APOE* *2, *3, and *4 polymorphisms were studied (Table 1). PCR amplifications were done according to Yasar et al. (1999) for *CYP2C9*2* and **3*, Adithan et al. (2003) for *CYP2C19*2*, and Dixit et al. (2005) for *APOE* polymorphisms. The primer sequences and restriction conditions for analyzing the polymorphisms are presented in Table 2. Electrophoresis was carried out on 6% (29:1) polyacrylamide gels, followed by silver staining.

Statistical analysis

Allele frequency counts were derived from the observed genotypes. The chi-square or the Fisher exact tests were used to compare discrete variables and to test the Hardy-Weinberg equilibrium. The Mann-Whitney U-test was conducted to compare the mean of dosage according to genotype. The clinical variables were correlated by the Pearson test (rho). Multiple regression analysis was conducted in order to predict the dosage according to the studied variables; this analysis

included variables with significant association in other reports. Probability values of less than 0.05 were considered statistically significant. The analysis was performed using SPSS version 15.0.

Table 1. *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *APOE**2, *3, and *4 polymorphisms.

Polymorphism	dbSNP ID	Base (amino acid change)	Locus	Effect
CYP2C9*2	rs1799853	430C>T (Arg144Cys)	10q24.1 exon 3	Lower enzyme activity
CYP2C9*3	rs1057910	1075A>C (lle359Leu)	10q24.1 exon 7	Lower enzyme activity
CYP2C19*2	rs4244285	681G>A	10q23.33 exon 5	Alternative splicing. Protein truncated
APOE*3[a]	rs429358/rs7412	112T/158T (Cys/Arg)	19q13.2	Wild
APOE*2	112T/158C (Cys/Cys)			Lower affinity to the receptor
APOE*4	112C/158T (Arg/Arg)			Higher affinity to the receptor

Arg = arginine; Cys = cysteine; Ile = isoleucine; Leu = leucine. [a]These allelic forms differ from each other only by amino acid substitutions at positions 112 and 158. According to http://www.cypalleles.ki.se/cyp2c9.htm, http://snpedia.com/index.php.

Table 2. Primer sequence and restriction conditions of the polymorphisms studied.

Polymorphism	Primer sequence (5' to 3')	Enzyme	Size of digested product (bp)
*CYP2C9*2*	TACAAATACAATGAAAATATCATG CTAACAACCAGACTCATAATG	*Ava*II (4 U/37°C overnight)	C:527 + 164 T: 691
*CYP2C9*3*	AATAATAATATGCACGAGGTCCAGAGGTA GATACTATGAATTTGGGACTTC	*Kpn*I (5 U/37°C overnight)	A: 141 C: 111 + 30
*CYP2C19*2*	AATTACAACCAGAGCTTGGC TATCACTTTCCATAAAAGCAAG	*Sma*I (5 U/30°C 1 h)	G:120 + 49 A: 169
*APOE**2-4	ACAGAATTCGCCCCGGCCTGGTACAC TAAGCTTGGCACGGCTGTCCAAGGA	*Hha*I (5 U/37°C 5 h)	*2: 91 + 83 *3: 91 + 48 + 35 *4: 72 + 48 +35 + 19

CYP2C9 *1 = allele C, *2 = allele T. *CYP2C9*1* = allele A, *3 = allele C. *CYPC2C19*1* = allele G, *2 = allele A. bp = base pairs. U = units of enzyme. Primer sequence is presented in forward and reverse consecutively.

RESULTS

In this study, a total of 71 patients (45 female, 26 male) receiving acenocoumarol were included and followed up by reviewing clinical records. The indications for anticoagulant therapy were categorized into two main groups: (1) prevention or treatment of thromboembolic diseases (20/71), such as deep vein thrombosis and pulmonary embolism; and (2) cardiovascular diseases (51/71), such as heart failure, prosthetic valve replacement, and aneurysm of the heart post heart attack. The demographic and clinical characteristics are presented in Table 3. The mean patient age and dose of acenocoumarol were 53.37 years and 2.38 mg/day, respectively. The patients of this study showed high body-mass indices.

The analysis of correlation (Table 4) showed that the *CYP2C19*2* genotype (44.2%, $P < 0.001$) and age (25.8%, $P = 0.03$) correlated negatively with the daily dose of acenocoumarol requirements.

Regarding the genotyping data, all loci agreed with the Hardy-Weinberg equilibrium expectations (Table 5). For the *CYP2C9* and *CYP2C19* polymorphisms, the homozygous polymorphic genotypes were not present in this sample tested. For *APOE* polymorphisms, the *2/*2, *2/*4, and *4/*4 genotypes were not found.

The interpopulation comparisons showed that the allele frequency of the *CYP2C9*2* polymorphism was much lower (7.8%, $P < 0.036$) when compared with Southern Europeans and Caucasians, but was higher than African and Eastern and South-Eastern Asian populations ($P <$

Table 3. Demographic and clinic characteristics of patients receiving acenocoumarol.

Variable	Mean ± SD
Age (years)	53.37 ± 17.46
Weight (kg)	69.81 ± 15.66
Height (m)	1.61 ± 0.09
BMI	26.88 ± 5.43
Body surface (m^2)	1.77 ± 0.27
INR	2.83 ± 1.99
Dose (mg/day)	2.38 ± 1.34

BMI = body mass index, INR = international normalized ratio, SD = standard deviation.

Table 4. Correlation analysis of the clinical variables contrasted with dose of acenocoumarol.

Variable	rho coefficient	P
*CYP2C9*2* genotype	-0.138	0.25
*CYP2C9*3* genotype	-0.214	0.08
*CYP2C19*2* genotype	-0.442	<0.001
APOE genotype	-0.059	0.62
Age	-0.258	0.03
Gender	0.172	0.15
Weight	0.021	0.86
Height	-0.058	0.63

Table 5. Genotype and allele frequencies of the polymorphisms studied.

Polymorphism	N (%)	P
*CYP2C9*2*		
Genotype		
*1/*1	60 (84.5)	0.79
*1/*2	11 (5.5)	
*2/*2	0 (0.0)	
Allele		
*1	131 (92.2)	
*2	11 (7.8)	
*CYP2C9*3*[a]		
Genotype		
*1/*1	63 (91.3)	0.93
*1/*3	6 (8.7)	
*3/*3	0 (0.0)	
Allele		
*1	132 (95.7)	
*3	6 (4.3)	
*CYP2C19*2*[a]		
Genotype		
*1/1	59 (84.3)	0.79
*1/*2	11 (15.7)	
*2/*2	0 (0.0)	
Allele		
*1	129(92.1)	
*2	11 (7.9)	
APOE		
Genotype		
*2/*2	0 (0.0)	0.98
*2/*34	2 (2.8)	
*2/*4	0 (0.0)	
*3/*3	58 (81.7)	
*3/*4	11 (15.5)	
*4/*4	0 (0.0)	
Allele		
*2	2 (1.5)	
*3	129 (90.8)	
*4	11 (7.7)	

P value calculated in Hardy-Weinberg equilibrium. [a]Some individuals were not included since it was not possible identify their genotype.

0.0005) (Kurose et al., 2012). The minor/major *2 allele frequency is described in the Eastern Asian (<0.1%) and Southern European (14.3%) populations, respectively (Kurose et al., 2012).

The allele frequency of *CYP2C9*3* (4.3%) was not different from the European, Caucasian, Asian, and African populations (Fisher exact test, P > 0.051). The minor *3 allele frequency has been described in the African population (1.8%) and the major allele in the Southern European (8.6%) population (Kurose et al., 2012). Both the *CYP2C9*2* and **3* allele frequencies were similar to that reported by Llerena et al., (2004) in a Mexican-American population (9 and 5%, respectively).

The *CYP2C19*2* allele had a frequency of 7.9%, similar (P ≥ 0.08) to Mexican-American, West Asian, and Southern and Eastern European populations (Luo et al., 2006; Kurose et al., 2012). The *CYP2C19*2* allele frequency was statistically different from Western and Northern European; Southern, South-Eastern, and Southern Asian; Caucasian; and African populations (P ≤ 0.042), whose frequencies range from 14 to 29.8% (Kurose et al., 2012).

Similar to other reports on the Mexican and Asian populations (Aceves et al., 2006; Al-Dabbagh et al., 2009), the *APOE* *3 allele had a frequency higher than 90% in this sample, unlike other ethnic groups where the *3 frequency ranges between 45 and 83% (Sconce et al., 2006; Kwon et al., 2010; Oriá et al., 2010).

The doses of acenocoumarol according to the genotypes are depicted in Table 6. The dose of this anticoagulant was statically different (P = 0.005) among *1/*2 and wild-type *CYP2C19* genotype carriers.

The multiple regression model used to predict daily dose requirements based on age and *CYP2C9* and *CYP2C19* genotypes is represented in Table 7. The variables of age and *CYP2C19*2* showed a modest significance.

Table 6. Dose requirements of acenocoumarol according to genotypes of CYP2C9, CYP2C19, and APOE polymorphisms.

Genotype	N (%)	Dose (mg/day) ± SD	P value	CI
CYP2C9[a]				
*1/*1	52	2.43 ± 1.33	-	-
*1/*2	11	1.89 ± 0.88	0.20	-0.29-1.39
*1/*3	5	1.44 ± 0.76	0.10	-0.22-2.21
CYP2C19*2[a]				
*1/1	59	2.56 ± 1.34	-	-
*1/*2	11	1.35 ± 0.84	0.005	0.37-2.05
APOE				
e2/e3	2	2.64 ± 0.91	0.77	-1.65-2.21
e3/e3	58	2.37 ± 1.35	-	-
e3/ε4	11	2.37 ± 1.46	0.99	-0.89-0.89

[a]Patients with morbid obesity or high INR were excluded. Wild type was considered as reference. SD = standard deviation; CI = confidence interval.

Table 7. Multiple regression model to predict daily dose requirements based on age, CYP2C9*2, CYP2C9*3, and CYP2C19*2 genotypes.

Variable	Univariated R^2	Combined R^2	B coefficient	CI	P
Constant	-	-	5.887	3.900-7.875	<0.001
Age	0.049	0.049	-0.018	-0.036-0.000	0.047
CYP2C9*2	0.109	0.158	-0.658	-1.456-0.141	0.105
CYP2C9*3	0.030	0.188	-0.823	-2.091-0.446	0.200
CYP2C19*2	0.022	0.210	-0.836	-1.655-0.018	0.045

CI = confidence interval. The formula to predict the doses was calculated as follows: Dose = 5.887 - 0.018 (1) - 0.658 (2) - 0.823 (2) - 0.836 (2) = 21%. Wild type is the unit meanwhile the heterozygous conditions are equivalent to twice.

DISCUSSION

Pharmacogenomics attempts to optimize and rationalize drug therapy according to genetic status, to ensure maximum efficacy with minimal adverse effects. In this and other fields, it is strongly recommended to evaluate the genetic attributes in the group of interest because of the associated inter- and intrapopulation variabilities, as seen in the principal genes associated with the metabolism of oral anticoagulants (Lee et al., 2007). Treatment with OAs are hampered because of their narrow therapeutic window, which is closely related to the individual genetic variability. In Mexico, acenocoumarol is the drug of choice for treatment of impaired coagulation, whereas warfarin seems to be preferred in many other countries around the world.

In this study, higher variations were detected in the mean dose of acenocoumarol (2.38 ± 1.34 mg/day) and the international normalized ratio (2.83 ± 1.99 mg/day). This could be explained by either a difference in the level of anticoagulation related with the disease or drug bioavailability interference factors such as age, gender, and allele variants.

There was an opposite relation between the *1/*2 *CYP2C19* genotype and age with the daily dose of acenocoumarol. This is consistent with the obtained results in the stratification of the mean dose of acenocoumarol according to genotype and the regression model where both variables are significantly involved.

As expected, the genetic distribution of every polymorphism tested showed higher variability when compared with other populations around the world.

The mean dose of acenocoumarol according to genotype stratification (Table 6) diminished more than 0.5 mg/day in *1/*2 and 1 mg/day in *1/*3 *CYP2C9* carriers, compared with the wild-type genotype; however, these results were not statically significant. The mean dose of acenocoumarol in *1/*2 *CYP2C19* carriers was lower than in *1/*1 carriers (1.35 *vs* 2.56 mg/day, P = 0.005). Several studies have analyzed the effect of polymorphisms in genes associated with the metabolism of acenocoumarol (Saraeva et al., 2007; Pérez-Andreu et al., 2010), finding significant association in *CYP2C9* mainly. However, only Saraeva et al., (2007) analyzed the *CYP2C19*2* polymorphisms, finding no significant results in the dose requirements of acenocoumarol. Our data suggest that *CYP2C19* could have a major participation in the metabolism of acenocoumarol compared with *CYP2C9* in the Mexican population tested. However, further studies including a larger sample size and other genes involved are desirable.

The *APOE* genotypes showed no statistical differences in mean doses, unlike others reports that have suggested an *APOE* contribution in dose requirement (Kohnke et al., 2005; Sconce et al., 2006). However, as stated above, the higher variability of *APOE* polymorphism among ethnic groups could have limited these results, given the lower frequency of *2 and *4 in our population.

The multiple regression model included the variables of age and *CYP2C9*2*, *CYP2C9*3*, and *CYP2C19*2*. These variables could explain 21% (Table 7) of the dose variations of acenocoumarol in the population tested. This value was small compared with other reports, which could be explained by the lower frequencies of *CYP2C9*2*, *CYP2C9*3*, and *CYP2C19*2* in the Mexican population.

To the best of our knowledge, there are no published studies in Mexico searching for the relationship in *CYP450* and *APOE* polymorphisms related with the dose requirements of acenocoumarol. The *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, and *APOE* *2, *3, and *4 polymorphisms showed higher frequencies of variability, compared with other ethnic groups, which

stresses the importance of evaluating for these alleles in the population of interest. *CYP2C19* heterozygous individuals (*1/*2) needed lower doses of acenocoumarol than wild-type carriers. Otherwise, the *CYP2C9* and *APOE* polymorphisms were not associated with acenocoumarol doses. Our results suggest that *CYP2C19* could have a much greater participation in acenocoumarol metabolism than *CYP2C9*, in the Mexican population. Besides considering the age factor, pharmacogenetic testing for *CYP2C19*2* prior to initiating acenocoumarol treatment could lead to a safer anticoagulation therapy in Mexican patients.

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