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Association of CYP2C19 genotype with type 2 diabetes

Carlos Hoyo-Vadillo[1]*, Jaime Garcia-Mena[2], Adán Valladares[3], Caterina R. Venturelli[1], Niels Wacher-Rodarte[4], Jesús Kumate[5], Miguel Cruz[3]

[1]Department of Pharmacology, Cinvestav-IPN Zacatenco, México City, Mexico; *Corresponding Author: citocromo@cinvestav.mx
[2]Departamento de Genética y Biología Molecular, Cinvestav-IPN Zacatenco, México City, Mexico
[3]Unidad de Investigación Médica en Bioquímica, Hospital de Especialidades, Centro Médico Nacional Siglo XXI del IMSS, México City, Mexico
[4]Unidad de Investigación Médica en Epidemiología, Hospital de Especialidades, Centro Médico Nacional Siglo XXI de IMSS, México City, Mexico
[5]Fundación IMSS, México City, Mexico

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ABSTRACT

Background: CYP2C19 is a major isoform of cytochrome P450 that metabolizes a number of commonly prescribed drugs such as omeprazole, diazepam, tolbutamide and propranolol. Its expression is regulated by the constitutive androstane receptor (CAR), involved in glucocorticoids synthesis. Since a number of crosslinks have been described for CYPs and some hormones, an association of CYP2C19 with type 2 diabetes is likely. Methods: Two groups were studied, 352 diagnosed with type 2 diabetes patients and 342 healthy volunteers form Mexico City. Both groups were tested for CYP2C19*2 and *3 alleles. We carried out an allelic discrimination using TaqMan assay for *2, and used FRET sensor and anchor probes for *3. Results: Ninety one percent of the subjects had the wild type allele, 9% have the *2 allele; no subject presented the *3 allele. The CYP2C19*2 allele is associated with type 2 diabetes (p = 0.012). Admixmap program was used to correct the admixture of this population and get the correlation. This was further confirmed in a linear model with a 67% power and by the method of Strom and Wienker for association on subjects within the mean range of Amerindian ancestry only (60%). Conclusion: Type 2 diabetes patients have significatly more *2 allele than healthy volunteers, more evident for the patients with the homocygous genotype.

Keywords: Pharmacogenomics; CYP2C19; Type 2 Diabetes; Mexicans; Allele Frequency

1. INTRODUCTION

The P450 Cytochrome gene super-family is involved in the synthesis of steroids, as well as in the metabolism of cholesterol [1]. Cytochrome genes exhibit a number of mutations that alter their activity [2]. In the case of CYP-2C19 the most common mutations *2 and *3 show no enzymatic activity. For this isoform, CYP2C19, known for metabolizing both, mephenytoin and omeprazole it has been demonstrated to have the greatest genetic variability among human populations [3]. Some of them, Asians for example [4], present a very high frequency of mutations, Latin-Americans on the other hand, have a small frequency of inactive alleles [5,6], while Eskimo Inuits do not seem to present any mutations at all [7].

CYP2C19 metabolizes an estimated of 8% of all therapeutic drugs in current use [8], knowing the variants which do not present enzymatic activity is of huge interest for physicians prescribing drugs such as tolbutamide, omeprazole, proguanil, fluoxetine, citalopram, diazepam, propanolol, diclofenac, indomethacine and others.

Previous studies have identified the presence of CYP2C19*2 in Bolivians [5] and in Colombians [6]. Whereas in Inuits no mutations have been identified [7]. Asians have a high frequency of 2C19 mutations, actually they are so far the only population with 2C19*3 mutations. For this reason it is somehow obvious to look for the presence of this allele in our Mexican subjects since Amerindian populations came from Asia 30,000 to 45,000 years ago [9]. In addition, characterizing our populations is an important advance towards a personalized medicine.

Nuclear receptors are transcription factors that modulate cellular responses to small lipophyllic molecules such as steroids and others. They also regulate the expres-

sion of CYPs. Three of this nuclear receptors have been described to regulate the expression of CYP2C19, CXR (chicken xenobiotic receptor), PXR (pregnane X receptor) and CAR [10]. All this elements are a linkage of CYP2C19 with its role on cholesterol elimination and on the physiology of other steroid hormones like cortisol. Both process are tightly involved in the development of obesity and type 2 diabetes. This regulation of the CYP2C19 is regulated by a complex crosslinking involving cholesterol levels, glucocorticoid activity and the action of other steroid hormones; therefore, the interest to look for a possible association of CYP2C19 with type 2 diabetes.

2. SUBJECTS, MATERIALS AND METHODS

2.1. Subjects

We included 367 men and 330 women (age range: 18-76 years old; body mass index averages of 28 for males and 30 for females, **Table 1**) from the DNA library of the Biochemistry Research Unit of Medical Center 21st century, IMSS, located in Mexico City. No relatives were included in the groups. The corresponding ethics and research committees approved the study. All participants signed the informed consent. About half of the subjects participating in this study were patients with type 2 diabetes, and because they were already part of other studies, we had previously characterized their population admixture.

2.2. Autosomal Ancestry-Informative Markers (AIMs)

Stratification in the subjects of this study was corrected using previously reported data [11], these markers have large frequency differences among populations of Native American (65%), European (30%), and West African (5%) ancestry.

2.3. Biochemical Profile

Clinical evaluation was made after 12 h overnight fasting. Blood samples were taken and used to measure glucose, triglycerides, total, high and low density cholesterol levels were assayed using an ILab 350 Clinical Chemistry System (Instrumentation laboratory, Mexico). All participants were interviewed by a physician who collected data about their weight, height, systolic and diastolic blood pressure. Quantitative measurements of fasting plasma insulin were carried out using the chemiluminescence's assay according to the manufacturer's instructions (Immulite, France).

2.4. DNA Samples

DNA was extracted from blood cells using the Qiagen columns according to the manufacturer's recommendations (Qiagen, Chatsworth, CA, USA). Purity was verified by UV absorption at 260/280 nm and DNA integrity was checked by electrophoresis in 0.8% agarose gels, stained with ethidium bromide.

Table 1. General characteristics of the group studied.

Variable	units	Control		T2D	
		Males	Females	Males	Females
Number		259	93	107	235
Age	years	41.6(8.2)	40.5(8.7)	57.8(9.9)	55.8(9.6)
Height	m	1.68(0.07)	1.56(0.06)	1.64(0.08)	1.54(0.07)
Weight	kg	77.9(10.5)	69.4(11.9)	76.1(11.3)	71.2(13.8)
Body Mass Index (BMI)	kg/m^2	27(3)	29(5)	29(5)	30(5)
Waist Hip Index		0.87(0.05)	0.84(0.07)	0.95(0.04)	0.89(0.07)
SBP	mmHg	118(11)	114(8)	122(12)	124(15)
DBP	mmHg	74(7)	73(8)	95(25)	92(24)
Glucose	mg/dL	85.7(9.5)	84(8.7)	163.5(73.3)	154.2(60.9)
Insulin	IU/ml	16(6.6)	15.6(7.6)	9.2(12.2)	8.4(11.5)
Glycosilated haemoglobin (A1c)	%	4.2(0.6)	4(0.5)	7.6(2.4)	7.9(2)
Total cholesterol	mg/dL	195(40)	189(36)	196(38)	206(46)
High density lipoprotein (HDL-C)	mg/dL	51(23)	59(23)	40(16)	42(19)
Low density lipoprotein (LDL-C)	mg/dL	106(46)	98(41)	119(39)	130(39)
Triglycerides	mg/dL	173(102)	128(62)	225(134)	207(127)
Serum Creatinine	mg/dL	1.00(0.16)	0.76(0.13)	0.93(0.98)	0.81(0.76)

Values are Mean (Standard Deviation).

2.5. Allele-Specific TaqMan PCR for CYP2C19*2

DNA samples were analyzed for CYP2C19*2 using the TaqMan PCR assay to detect G > A polymorphism according to the manufacturer's instructions. Amplification and detection were made using the ABI PRISM 7000 (Applied Biosystems, USA) system with the following profile: a denaturing cycle of 95ºC for 10 min and 40 cycles of 92ºC for 15 s, extension phase of 60ºC for 1 min. Samples were judged positive for *2 when the value of the emitted fluorescence was greater than the threshold calculated by the instrument's software. Displayed as an allelic discrimination plot. Wild type allele CYP2C19*1 was labeled with VIC and *2 allele with FAM. VIC was for TTCCC**G**GGAACCCA and FAM for ATTTCCC**A**GGAACCCA (SNP showed in boldface).

2.6. CYP2C19*3 Evaluation by FRET Technology

This polymorphism was identified following the method described by Borlak and Thum [12] using fluorescence resonance energy transfer (FRET) to assay genomic G > A Sensor and anchor probes were labeled with fluorescein and Red640, respectively. PCR was run with a 5 min denaturation step. Fifty cycles with 7 sec of annealing (48ºC) and 14 sec of extension (72ºC) in a Light Cycler (Model 1.2, Roche diagnostics, Germany). The melting curve was done from 40ºC (with 30 sec of previous stabilization) to 80ºC with a 0.1ºC/min slope. Tms were 61ºC for the wild CYP2C19*1 type and 67ºC for the CYP2 C19*3 genotype. Software used was Light Cycler (Roche, Version 4). Control probes for *1 and *3 were used as validation of fusion points. The assay was validated using standards provided by Roche.

2.7. Statistical Analysis

In order to correct the stratification of this population, ADMIXMAP program was employed to look for case-control (shown in **Table 2**) associations using logistic regression and to measure the Hardy-Weinberg equilibrium. Iterations were tested to 4000, burning was selected to 200, "every" parameter to 7. The rest of parameters were defaults. Co-variables were log of age and gender. Hardy-Weinberg equilibrium was also tested after the Sasieni method implemented by Strom and Wienker [13] (available at: http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) [14]. Proportions of genotypes were compared by the Fisher exact test. Odds ratios and their corresponding 95% confidence intervals were also estimated. All p values < 0.05 were considered statistically significant. The association between total cholesterol, HDL-C, LDL-C levels with the CYP2C19*2 allele was analyzed by the type III linear model with and without co-variables, including the stratification of the population, health, age, gender, height, body mass, waist-hip index, and BMI. Linear models were also tested using model I for interaction, with an Amerindian index as co-variable as well as log of age and gender. In all tests significance was considered for values less than 0.05.

3. RESULTS

3.1. Characteristics of the Sample and Stratification of the Population

At total of 697 individuals were studied (**Table 1**). This sample of Mexico City population had been previously characterized for its ancestry informative makers by admixture mapping analysis, showing an estimated proportion of 65% component of Amerindian, 30% European

Table 2. Linear model for CYP2C19 associtation with type 2 diabetes.

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power(a)
Corrected Model	48.1(b)	4	12,042	87,292	0.000	349,169	1,000
Intercept	94,616	1	94,616	685,863	0.000	685,863	1,000
Homocygotic	0.8	1	0.800	5800	0.016	5,800	0.671
Amerindian index	0.186	1	0.186	1,346	0.247	1,346	0.212
Sex	16,589	1	16,589	120,254	0.000	120,254	1,000
In (Age)	30,593	1	30,593	221,769	0.000	221,769	1,000
Error	58,216	422	0.138				
Total	201,000	427					
Corrected Total	106,384	426					

a: Computed using alpha = 0.05; b: r Squared = 0.453 (Adjusted R Squared = 0.448).
Homocygotic referes to 2,2 genotype for CYP2C19.
Amerindian index was obteinded from admixmap and equals the integer of ameriandian component multiplied by 10 and divided by 4.

and 5% West African. For this study, the stratification plays a critical role since the frequency of CYP2C19 is biased by ethnic admixture.

3.2. Genotypes

CYP2C19*2 genotypes were characterized for all subjects. In our control subjects, 0.6% were homozygous and 15.6% heterozygous for *2. In T2D patients, 1.4% were homozygous and 17.7% heterozygous for *2. These frequencies were in Hardy-Weinberg equilibrium after Fisher exact test (p = 1.000 for the control volunteers and p = 0.384 for the T2D group. CYP2C19*3 genotypes were not present in none of analyzed samples. The genotype frequencies are shown in **Figure 1**.

3.3. Allele Frequencies

Eight percent of the alleles in the control subjects, and 10% in T2D patients were CYP2C19*2 (**Table 3**). The odds ratio was 1.22, with 95% confidence interval [0.81-1.84], (p = 0.358), in a dominant model; and 2.57 with a 95% confidence interval of [0.44 - 19.26], (p = 0.431) in a recessive model. The CYP2C19*3 allele was analyzed in 460 subjects: 198 control volunteers and 262 type 2 diabetes patients and all of them had the wild type form for the loci 636G > A (**Table 2**).

3.4. There is No Association between the CYP2C19*2 Allele and HDL-C, LDL-C and Total CYP2C19 Association with Type 2 Diabetes

Admixmap program revealed a significance of 0.012 for association of CYP2C19 genotypes with type 2 diabetes. The power for this test is indirectly estimated to be 0.95, using the G*power program. This was confirmed using the linear model I. In this case ethnicity measured as Amerindian content in a 1 to 4 scale. This index was calculated by scaling the results of Amerindian ancestry. Log of age and gender as co-variables were also included. The significance was of 0.016 for subjects with recessive homozygous genotype. The statistical power for this test was 0.67. When subjects with Amerindian index of 3 were selected, an association was detected using the Sasieni procedure; however in this case type 2 diabetes subjects were not in Hardy-Weinberg equilibrium. Odds ratio for that group is shown in **Table 4**.

Classical approach using linear model also show the association for CYP2C19 with type 2 diabetes, see **Table 2**. In that case age and sex were taken as covariables, and the significance was 0.016.

4. DISCUSSION

Our finding of the *2 allele association with type 2 diabetes is apparently due to the homozygous genotype group as seen in the raw database. Of six subjects presenting this genotype five were type 2 diabetes patients versus only one in the control group. The only healthy *2/*2 carrier was a young male (40 years) who is probably in high risk for developing type 2 diabetes. Type 2 diabetes is a multifactor disease; the role of CYP2C19 in this pathogenesis can be linked by its interaction with CAR and PXR [15,16]. Interestingly Kohalmy [17] have described a CYP2C19 relationship with DHEA which at the same time is related to type 2 diabetes. Type 2 diabetes patients present increased CYP2E1 activity, measured as a decrease of the area under the curve after chlorzoxazone administration. CYP2E1 mRNA, in blood mononuclear cells, was found increased as well [18]. On the other hand, it has been previously suggested that free radicals are a risk factor for type 2 diabetes. One source for the suppression of free radicals happens to be CYP activity. Among the involved isoforms, the CYP2C family can play a major role [19].

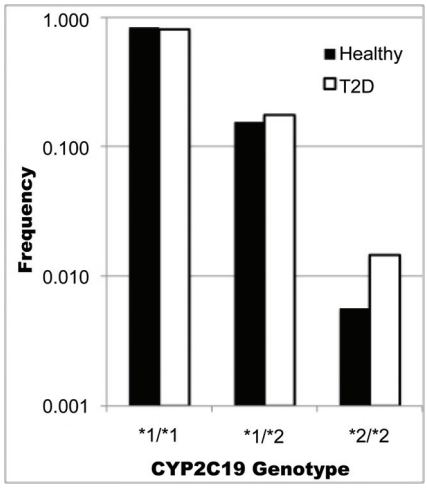

Figure 1. Observed frequency of CYP2C19 genotypes on case (T2D) and control (healthy) groups. Note that the slight higher frequency of *1/*2 genotype together with the much higher frequency of *2/*2 in case group are responsible for the association of CYP2C19 genotype with type 2 diabetes (T2D).

It has to be pointed out that CYP2C19*2 comprises an haplotype with at least four mutations: 99C > T; 681G > A; 990C > T; 991A > G [20]. It has four variants: 2A, 2B, 2C and 2D; the last three, besides the mentioned four mutations also have other substitutions: 276 G > C, 481 G > C and 1213 G > A, respectively. For CYP2C19*2 the 681G > A, SNP was included in the taqman assay. CYP2C19*3 have two haplotypes: 3A (636G > A; 991A > G; 1251A > C) and 3B (636G > A; 991A > G; 1078G > A; 1251A > C) [20]. For CYP2C19*3 the SNP of 636 G > A was the one included for the Light Cycler system.

Table 3. Observed frequencies of CYP2C19*2 and *3.

	Healthy	T2D	Healthy	T2D	Healthy	T2D
Genotype	**Number of subjects**		**Observed Frequency**		**H-W Expected freq.**	
*1/*1	295	279	83.8%	80.9%	83.9%	80.5%
*1/*2	55	61	15.6%	17.7%	15.4%	18.5%
*2/*2	2	5	0.6%	1.4%	0.7%	1.1%
*1/*3	0	0	0%	0%	0%	0%
*2/*3	0	0	0%	0%	0%	0%
*3/*3	0	0	0%	0%	0%	0%
Allele *1					91.6%	89.7%
Allele *2					8.4%	10.3%
Allele *3					0%	0%

Table 4. Odds ratio for genotypes in healthy males (control) versus diabetic males.

Risk allele 2				
[1]<->[2]	[11]<->[12]	[11+]<->[22]	[11]<->[12+22]	Common odds ratio
Odds_ratio = 1.992	Odds_ratio = 1.724	Odds_ratio = 5.663	Odds_ratio = 1.907	Odds_ratio = 2.741
C.I. = [1.084-3.659]	C.I. = [0.904-3.289]	C.I. = [0.310-103.496]	C.I. = [1.006-3.617]	
chi2 = 5.09	chi2 = 2.78	chi2 = 2.54	chi2 = 4.00	chi2 = 4.88
p = 0.02404 (Pearson)	p = 0.09571	p = 0.11067	**p = 0.04543**	**p = 0.02711**
Risk allele 1				
[2]<->[1]	[22]<->[12]	[22]<->[11]	[11+12]<->[22]	Common odds ratio
Odds_ratio = 0.502	Odds_ratio = 0.298	Odds_ratio = 0.177	Odds_ratio=0.193	Odds_ratio=0.543
C.I. = [0.273-0.922]	C.I. = [0.016-5.714]	C.I. = [0.010-3.227]	C.I. = [0.011-3.522]	
chi2 = 5.09	chi2 = 1.46	chi2 = 2.54	chi2 = 2.33	chi2 = 4.88
p = 0.02404 (Pearson)	p = 0.22749	p = 0.11067	p = 0.12663	**p = 0.02711**

For both alleles just one SNP was included as the standard technique proceeds, because SNPs always come together for a given haplotype, in other words, one SNP, the one shared for all variants, is enough to characterize the haplotype.

Complex networks of nuclear receptors, where PXR and CAR are outstanding representatives, regulate the expression of CYP3A4 and CYP2C19. The latter is modulated by glucocorticoids which at the same time interact with insulin for energy balance altogether with the participation of the neuropeptide Y.

Since it has been demonstrated that CAR also regulates the expression of CYP2C19 [21] the CYP2C family can be considered part of the xenosensing mechanism in charge of the regulation of cholesterol, bile acids and indirectly the uptake of dietary cholesterol as Handschind [16] describes it. Furthermore, the crosstalk between all the orphan nuclear receptors has been pointed out by several authors [22,23]. After all CYPS are not very substrate specific and the interesting conclusion of the Meyer group that the organism manages xenobiotics and drugs as toxic bile salts [24]. These changes suggest a crosstalk between the factors deriving in type 2 diabetes and the regulation of cytochromes P450. At the moment the regulation of CYP2C19 has been demonstrated but the inverse can be only assumed as part of a feedback system.

In conclusion our work shows that the mexican popu-

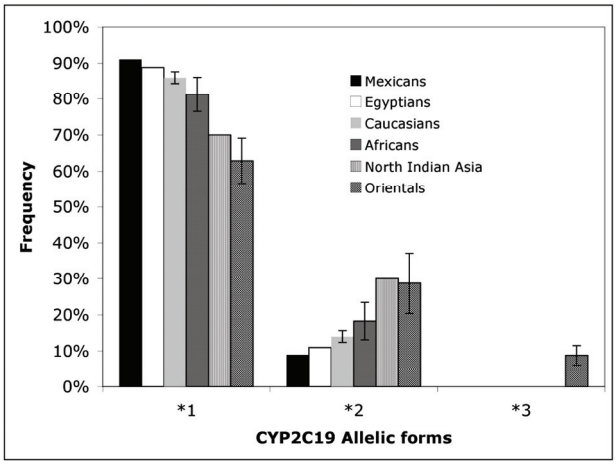

Figure 2. Frequencies of main alleles of CYP2C19 on different populations. Colombians, Bolivians and Mexicans have the lower frequencies for *2 allele. But Inuits have no presence of them. While Asians have the greatest frequencies for the *2 and only they present *3.

lation of Mexico City has the same frequencies for CYP2C19 wild and *2 alleles than the populations reported in Bolivia and Colombia (**Figure 2**). The *3 allele was not detected in our population just as in other Latin-Americans.

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