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Assessment of the rs4340 *ACE* gene polymorphism in acute coronary syndrome in a Western Mexican population

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ABSTRACT. Acute coronary syndrome (ACS) is considered one of the main causes of death worldwide. Contradictory findings concerning the impact of the angiotensin-converting enzyme (*ACE*) gene on cardiovascular diseases have been reported. Previous conclusions point out that the variability in results depends on ethnicity and genetic polymorphisms to determine the association of rs4340 polymorphisms of the *ACE* gene and ACE circulating levels in ACS. Genotyping of rs4340 polymorphisms was performed in a total of 600 individuals from Western Mexico divided into two groups: the ACS and the control group (CG). The polymorphisms were identified by polymerase chain

reaction. Serum ACE concentration was determined by enzyme-linked immunosorbent assay. D/D carriers had higher ACE levels than I/I carriers (3.6 *vs* 2.8 ng/mL, $P < 0.0021$) in the CG. The D/D genotype of the rs4340 polymorphism is associated with higher ACE concentration levels; however, the polymorphism was not associated with ACS.

Key words: Acute coronary syndrome; Angiotensin-converting enzyme; Serum concentration; Genetic polymorphisms; Western Mexican population

INTRODUCTION

Acute coronary syndrome (ACS) is considered one of the main causes of death and emergency-service issues worldwide (WHO, available at http://www.who.int/mediacentre/factsheets/fs310/en, accessed June 15, 2016). The ACS clinical spectrum includes the ST-segment elevation myocardial infarction (STEMI), the non-ST-segment elevation myocardial infarction (NSTEMI), and unstable angina (UA) (Rajpurohit et al., 2015). The pathophysiology of ACS involves thrombotic, inflammatory, oxidative, and metabolic processes. Infiltrating inflammatory cells interact with the intrinsic arterial cells, promoting lesion formation and complications (Libby et al., 2011). The angiotensin-converting enzyme (*ACE*) gene contributes widely to atheroma formation, inflammatory process, and thrombosis, leading to acute myocardial ischemia (Curzen and Fox, 1997).

The *ACE* gene is located on chromosome 17q23 and contains 26 exons and encodes two enzymes, namely, sACE (somatic) and tACE (testicular). The ACE protein belongs to the dipeptidyl-carboxypeptidases of the metalloprotease family, which is involved in the conversion of the angiotensin I (Ang I) decapeptide into octapeptide angiotensin II (Ang II). The latter is a powerful vasopressor that regulates blood pressure and salt-water homeostasis, mainly through aldosterone biosynthesis (Hubert et al., 1991). ACE is shed from the cell surface by a disintegrin and metalloproteinase domain-family alpha secretase, releasing the soluble form. In sACE, the cleavage site is arginine 1203 and serine 1204, 24 residues proximal to the membrane-anchoring domain (Parkin et al., 2004). Serum ACE concentration in normal subjects is reported within a wide range, being affected by age, gender, and ethnicity (McKenzie et al., 1995; Villard et al., 1996; Fagyas et al., 2014). Some polymorphisms could alter the transcription rate and serum concentration of protein, thus modifying ACS susceptibility. The 287-bp insertion/deletion ACE polymorphism (rs4340) is located in the 16th intron of the *ACE* gene. D-allele carriers have demonstrated increased Ang I-Ang II conversion and ACE concentration, with an increased predisposition for myocardial infarction and coronary heart disease (Cambien et al., 1994; Kondo et al., 2015). To the best of our knowledge, this polymorphism has not been studied regarding ACS susceptibility in our population.

MATERIAL AND METHODS

Genotyping was performed in 300 ACS patients and 300 healthy individuals (the control group, CG). Patients with ACS were diagnosed by the criteria of the American College of Cardiology (ACC) (Cannon et al., 2013). Classical risk factors, defined according to the ACC, were categorized as present or absent. The inclusion criteria for CG were the absence of ischemic cardiopathy and infectious diseases. The controls were similar in age to the cases,

without medical treatment. Subjects were recruited from the Hospital de Especialidades del Centro Médico Nacional de Occidente del Instituto Mexicano del Seguro Social (CMNO, IMSS). Only individuals who had been born in Western Mexico and for three generations including their own were considered.

All subjects and patients agreed to participate in the study and signed an informed written consent. The study was performed following the ethical guidelines of the 2013 Declaration of Helsinki and with the approval of the Ethics Committee of the Centro Universitario de Ciencias de la Salud (CUCS), Universidad de Guadalajara (UdeG). Genomic DNA was extracted from peripheral blood according to the salting out method (Miller et al., 1988). The analysis was performed with polymerase chain reaction (PCR); this polymorphism was subjected to two rounds of amplification. The primer sequences were the following: forward (5'-CTG GAG ACC ACT CCC ATC CTT TCT-3') and reverse (5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3'). The presence of allele I or D resulted in a 490- or a 190-bp fragment. PCR amplification was carried out in a total volume of 15 µL containing 5 µg genomic DNA, 1.25 U/L Taq DNA polymerase (Invitrogen Life Technologies), 1X supplied buffer enzyme, 20 µM of each oligonucleotide, 2.5 mM $MgCl_2$, and 2.5 mM dNTP (Invitrogen Life Technologies). Thermocycling conditions had an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 20 s each at 94°, 62°, and 72°C, with a final extension step of 10 min at 72°C.

Due to preferential amplification of the smaller allele (D), D homozygous carriers underwent a second amplification with the following insertion-specific primers: forward (5'-TGG GAC CAG AGC GCC CGC CAC TAC-3') and reverse (5'-TCG CCA GCC CTC CCAT GCC CAT AA-3') to discriminate heterozygotes, obtaining a 335-bp band if one allele was present. For amplification control in this assay, tumor necrosis factor alpha (TNF-α) primer sequences were utilized: forward (5'-TAT GTG ATG GAC TCA CCA GG-3') and reverse (5'-CCT CTA CAT GGC CCT GTC TT-3'); the amplicon was 264 bp. PCR was carried out in a total volume of 15 µL containing 5 g genomic DNA, 1.25 U/µL Taq DNA polymerase (Invitrogen Life Technologies), 1X supplied buffer enzyme, 20 µM of each oligonucleotide, 2.5 mM $MgCl_2$, and 2.5 mM dNTP (Invitrogen Life Technologies). Thermocycling conditions comprised an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 20 s each at 94°, 66°, and 72°C, with a final extension step of 1 min at 72°C. The amplicon was visualized by silver-stained polyacrylamide gels (29:1). ACE levels were measured in duplicate using serum using the enzyme-linked immunosorbent assay according to the manufacturer's specifications (R&D Systems, Minneapolis, MN, USA). The ACE range was 156-10,000 pg/mL, and the sensitivity of the assay was <5 pg/mL. The ACE concentration was calculated using a four-parameter logistic (4-PL) curve fit. The coefficients of variation fell within acceptable ranges (<10%).

The SPSS statistical software package version 20.0 was employed for statistical analysis. Continuous variables are reported as means ± standard deviation (SD). The Kolmogorov-Smirnov test and sample size were considered to categorize parametric tests (Student *t*-test and analysis of variance) and non-parametric tests (Spearman correlation, Kruskal-Wallis test, and Mann-Whitney U-test). Qualitative data and Hardy-Weinberg equilibrium were analyzed with the chi-square or the Fisher exact test, when applicable. Allele frequencies were determined by the counting method. Recessive allele model was tested with the chi-square test. The significance level was <0.05 (Lander and Kruglyak, 1995). The measurement of association was the odds ratio.

RESULTS

CG subjects and patients with ACS had a mean age of 55.4 and 62.7 years, respectively (Table 1). For the ACS group, the male was three times more frequent than female gender; cardiac biomarker values, including troponin I, creatine phosphokinase (CPK), and CPK-MB were increased. Similarly, plasmatic glucose was above normal values. The most prevalent risk factor was hypertension, followed by type 2 diabetes mellitus (DM2) and smoking. Treatment included administration of antiplatelet (acetylsalicylic acid, clopidogrel), heparin, statins (atorvastatin), and antihypertensive drugs (Table 2).

Table 1. Demographic and biochemical parameters of acute coronary syndrome (ACS) patients and control group (CG).

Parameters	ACS	(Average ± SD)	CG	(Average ± SD)	Normal range
Ratio (M/F)	3.21		1.09		
Age (years)	62.7	± 10.9	55.4	± 10.9	
Glucose (mg/dL)	138.5	± 57.7	117.5	± 87.2	70-100
Cholesterol (mg/dL)	118.9	± 39.1	165.6	± 43.6	150-199
Triglycerides (mg/dL)	91.9	± 35.3	116.3	± 63.0	<200
LDL (mg/dL)	45.3	± 19.1	73.8	± 32.3	<130
HDL (mg/dL)	21.0	± 11.9	40.5	± 20.0	>40
CPK (µ/L)	859.9	± 1403.6	-	-	2.4-19.5
CPK-MB (µ/L)	108.9	± 160.8	-	-	49-397
Troponin I (ng/ml)	7.1	± 14.5	-	-	<0.04

LDL: low-density lipoprotein; HDL: high-density lipoprotein; CK: creatine phosphokinase; CK-MB: creatine phosphokinase MB; SD: standard deviation; M: male; F: female.

Table 2. Medical treatment and risk factor prevalence in the ACS patients.

Treatment	N	(%)	Risk factor	N	(%)
ARA-II	55	(16.5)	IFH	145	(42.4)
Acetylsalicylic acid	326	(95.3)	Dyslipidemia	147	(43.6)
CCB	41	(12.3)	DM2	178	(52.0)
BB	196	(57.3)	COPD	3	(0.9)
Clopidogrel	241	(72.2)	HBP	226	(66.1)
Statins	269	(80.5)	RHA	51	(14.9)
Heparin	262	(79.9)	Obesity	92	(26.9)
ACE inhibitors	189	(56.6)	Overweight	139	(40.6)
Nitrates	146	(43.6)	Smoking	172	(50.3)

ARA-II: receptor antagonists of angiotensin II; CCB: calcium channel blockers; BB: beta blockers; IFH: inherited family history; DM2: type 2 diabetes mellitus; COPD: chronic obstructive pulmonary disease; HBP: high blood pressure, RHA: recurrent heart attack.

The genotype distribution was performed by Hardy-Weinberg equilibrium expectations ($P = 0.64$). Allele and genotype frequencies were similar between groups (Table 3). D allele frequency was 59.6% in the CG and 53.9% in ACS. In the recessive model, a discrete difference was found (OR = 1.445; $P = 0.044$). The risk for D allele prevalence in the CG was similar ($P > 0.01$) compared with other populations such Mexican Pima (46%), European (51%), and Hispanic from the USA (45%) although different from African (75%), Asian (72%), and Yucatec Mayas (27%) (http://alfred.med.yale.edu/alfred; accessed June 10, 2016).

Table 3. Allele and genotype distribution of the ACE polymorphism.

11689 I/D (rs4340)	ACS N = 300 (%)	CG N = 300 (%)	OR* (CI)	P value
Allele				
I	277 (46.1)	301(40.4)	-	
D	323 (53.9)	444 (59.6)	1.174(0.936-1.472)	NS
Genotype				
I/I	71 (23.6)	73 (24.0)	-	-
I/D	135 (45.0)	155 (51.7)	0.896(0.600-1.336)	NS
D/D	94 (31.3)	72 (24.0)	1.342(0.857-2.105)	NS
Recessive model				
I/D + D/D	229 (76.3)	227 (75.7)	-	-
I/I	71 (23.7)	73 (24.3)	1.445(1.008-2.071)	0.044

CG: control group; ACS: acute coronary syndrome; OR: odds ratio; CI: confidence interval.

The median ACE concentration was similar between ACS and CG (3.5 *vs* 3.04 ng/mL). Likewise, the ACE concentration was similar to clinical spectrum in patients (Figure 1). Neither age (less or higher than 55 years) nor gender affected the ACE concentration.

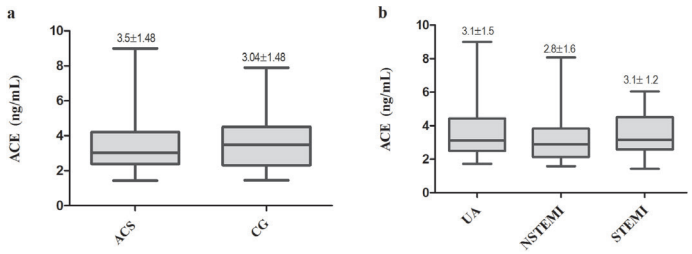

Figure 1. ACE serum concentration in the study groups and ACS clinical diagnosis. **a.** ACE concentration by the studied group. **b.** ACE concentration by clinical diagnosis in ACS. ACE: angiotensin-converting enzyme; ACS: acute coronary syndrome; CG: control group; NSTEMI: non-ST-segment elevation myocardial infarction; STEMI: ST-segment elevation myocardial infarction; UA: unstable angina. Boxes indicate the interquartile range (IQR) with the median; whiskers are minimal and maximal values. Medians (numbers above the upper bar) were compared by the Mann-Whitney U-test.

To test the influence of the polymorphisms on ACE concentration, we stratified data according to genotypes in both groups. The D/D homozygous displayed higher ACE median concentrations than those in I/I carriers (3.6 *vs* 2.8 ng/mL, $P < 0.0021$) in the CG (Figure 2a), while in patients, the median ACE concentration was similar by genotype (Figure 2b).

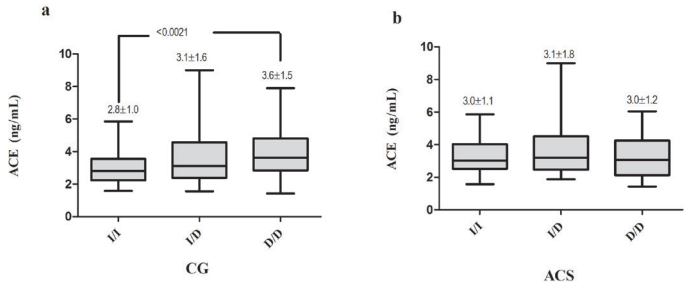

Figure 2. Comparison between ACE genotypes and ACE serum concentration. **a.** Influence of the rs4340 polymorphism on ACE concentration in the CG. **b.** Influence of the rs4340 polymorphism on ACE concentration in ACS. ACE: angiotensin-converting enzyme; ACS: acute coronary syndrome; CG: control group. Boxes indicate the interquartile range (IQR) with the median; whiskers are minimal and maximal values. Medians (numbers above the upper bar) were compared by the Mann-Whitney U-test.

DISCUSSION

Contradictory findings concerning the impact of the *ACE* gene on cardiovascular diseases have been reported. The main prior conclusions point out that the variability in results depends on ethnicity and genetic polymorphisms (Sayed-Tabatabaei et al., 2006). The main risk factors in our patients with ACS were hypertension (66.1%), smoking (50.3%), DM2 (52%), and dyslipidemia (43.6%). Previous reports described the rise of hypertension in patients with myocardial infarction and even an increase in younger age groups (INEGI, available at http://www.inegi.org.mx/inegi/contenidos/espanol/prensa/Contenidos/estadisticas/2013, accessed May 30, 2016; García Castillo et al., 2005). As can be noted, the risk factors are present in close to or more than one-half of the cases, contributing to a propitious environmental framework in its development. Biochemical parameters revealed a slight elevation of glucose; this increase has been attributed to the activation of inflammatory mediators, such as nuclear factor-kappa beta, which induces a transient increase during the acute event (Arnold et al., 2014). CCPK and Troponin I values were above the normal range (>44 times), which is expected in that they are a core part of the pathology diagnosis. Due to its high cardiospecificity, Troponin I has become the cornerstone for risk stratification in ACS because the remainder of cardiac biomarkers can be altered by other conditions involving muscle injury or trauma (Cengiz et al., 1991; Tang et al., 2008).

A previous study in Mexicans with hypertension without ACE inhibitors reported a serum concentration of ACE between 2.6 and 3.8 ng/mL, stratified by low- and high-haplotype risk, respectively (Martínez-Rodriguez et al., 2013). These concentrations are nearly similar to those reported in the present study; however, the study design was different from ours, and none of the polymorphisms studied in our study were included. We had clear results that serum ACE concentration in ACS is biased by ACE inhibitors and Ang II receptor antagonists (ARAII); however, ethical principles would not allow us to withdraw consumption of the drug from our patients. Intriguingly, modifiers of ACE treatment did not show the enzyme concentration. It is probable that the sample size of patients who were not receiving these drugs does not permit us to conduct a reliable analysis; consequently, this factor is an intervening variable that should be considered in future studies.

Regarding the genetic association of this polymorphism with ACS, we did not find evidence of such an association. Conversely, Chen et al. (2012) found the D/D genotype associated with ACS and disease severity in Taiwan population. Besides, other studies have demonstrated a consistent association of the polymorphism with myocardial infarction or coronary artery disease (Keavney et al., 2000; Kaiser Jamil, 2009; Vaisi-Raygani et al., 2010). The relationship of this polymorphism with cardiovascular disease has been correlated with its functional effect. The D allele is proposed to remove a gene expression repressor site; thus, the carriers of this allele have higher ACE levels, which are linked with adverse cardiovascular effects (Rosatto et al., 1999). Rigat et al. (1990) reported that ACE activity is proportional to the serum ACE concentration and that the rs4340 polymorphism accounts for one-half of the variance observed in ACE serum levels. Similar to that previously described, we found that D/D carriers had higher ACE concentration than I/I genotype in controls. With these results, we confirmed that the D allele determines higher concentrations of the enzyme, although we were not able to confirm the increased ACS risk regarding this polymorphism.

In conclusion, the D/D genotype of the rs4340 polymorphism is associated with higher ACE concentrations; however, the polymorphism is not a risk factor for the development of ACS in a western Mexican population.

Conflicts of interest

The authors declare no conflict of interest.

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