UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

PROGRAMA DE MAESTRÍA Y DOCTORADO EN CIENCIAS MÉDICAS, ODONTOLÓGICAS Y DE LA SALUD

INSTITUTO NACIONAL DE CARDIOLOGÍA IGNACIO CHÁVEZ

CAMPO DE CONOCIMIENTO DE CARDIOLOGÍA

THE EDN-1 GLU105GLU (RS5369) GENE POLYMORPHISM IN THE ENDOTHELIN GENE (EDN1) IS ASSOCIATED WITH RISK OF DEVELOPING CORONARY ARTERY DISEASE IN MEXICAN PATIENTS.

MODALIDAD DE GRADUACIÓN: PRODUCCION CIENTÍFICA

QUE PARA OPTAR POR EL GRADO DE MAESTRO EN CIENCIAS MEDICAS

PRESENTA

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PROGRAMA DE MAESTRÍA Y DOCTORADO EN CIENCIAS MÉDICAS, ODONTOLÓGICAS Y DE LA SALUD

CIUDAD DE MEXICO, FEBRERO DEL 2017

“Noli foras ire, in te ipsum redi, in interiore homine habitat veritas”

Sant’Agostino

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1. INTRODUCTION

1.1 From the endothelial dysfunction to the atherosclerotic process

Atherosclerosis is a complex multifactorial and polygenic disorder resulting from endothelial dysfunction (ED) (1) and peculiar responses to various forms of injurious stimuli to the arterial wall (2).

The latest observations suggest that plaque rupture (PR) represent the most common substrate of coronary thrombosis (in nearly 50% of cases) (3,4), whereas coronary erosion is responsible for 25-30% of acute coronary syndromes (ACS) (5). A recent study showed that patients with PR as culprit lesion present a poor prognosis compared to patients with intact fibrous cap (6). Mechanisms of PR have been mainly focused on inflammation as plaque inflammation may predispose to PR by thinning of the fibrous cap due to release of metalloproteinase and to reduced collagen synthesis (7). This hypothesis is in keeping with data showing higher values of C-reactive protein (CRP) a sensitive marker of inflammation in patients with ACS as compared to patients with chronic stable angina (8). Yet, CRP levels are normal in about a half of ACS patients (9), suggesting that inflammation is probably involved in PR in a subset of patients.

On the other hand, previous studies discovered several molecules that have been associated with the vascular physiology and severity of coronary diseases (10), the most important of them are represented by Endothelin-1 (ET-1) and endothelial nitric oxide (eNOS).

1.2 Endothelin-1

Endothelin (ET-1) is encoded by the EDN1 gene located in chromosome 6p21–24, and acts as a potent vasoconstrictor and modulator of vasomotor tone and vascular remodelling (11,12). Moreover, ET-1 has the following properties: 1) it is expressed in several tissues, including endothelial cells and cardiomyocytes (13); 2) it has a pro-atherogenic activity and mediates smooth muscle cell proliferation via ET-1 receptors; 3) it also acts as a chemoattractant for monocytes, and induces platelet aggregation and expression of adhesion molecules (14-16); 4) finally, it stimulates superoxide production, NF-κB expression, and atherosclerotic lesion formation, and inhibits endothelial nitric oxide (eNOS) (17).

1.3 Endothelian nitric oxide.

Another crucial actor of the atherosclerotic process is represented by eNOS. The gene encoding eNOS is located on chromosome 7q35–36. It is considered a protective enzyme, not only for its role in NO synthesis but also because its inhibition is associated with the production of superoxide (18,19). Yet, eNOS regulates a wide spectrum of functions in the cardiovascular system, like vasorelaxation and migration and proliferation of vascular smooth muscle cell (20). Of note, it inhibits T helper 1 type immune response and can activate directly endothelial NADPH oxidases, (21). For these reasons, several studies have assessed the correlation between endothelial expression levels of ET-1 and eNOS with the instability of the atherosclerotic plaque (22-24).

1.4 Single-nucleotide polymorphisms.

The human genome is rich in variation. These variants include single nucleotide substitutions (mutations or single nucleotide polymorphisms), insertions and deletions

(indels), copy number variants, and short tandem repeats. In many cases, the genetic variant detected is a marker for another genetic defect at a nearby locus rather than a cause of the phenotypic abnormality. Single nucleotide polymorphisms (SNPs; ie, substitutions of one nucleotide for another) represent the most abundant form of genetic variation and are responsible for much of the heritable phenotypic variation observed in human populations. Estimates based on whole genome sequencing efforts suggest that individuals carry between 2.8 and 3.9 million single-base pair variants, with higher estimates observed in individuals of African descent.

Estimates suggest that unrelated haploid genomes differ every 185 to 2000 nucleotides. This wide range is due in part to the variable rate of polymorphism across the genome, with some regions demonstrating high heterozygosity (such as the MHC cluster on chromosome 6), while others display more restricted variation.

By convention, single-base pair changes that achieve a population frequency of at least 1 percent are referred to as SNPs. Less-frequent substitutions that interrupt gene function, or that have clinical consequences, are labeled as mutations, even though many of these are observed at a fixed frequency (albeit below 1 percent) across diverse populations. Differences in the frequency distribution of SNPs result from the combination of effects of natural selection, genetic drift, and other population genetic effects. The functional impact of a variant is not determined by its frequency, but rather by its location within genes or non-genic regulatory regions. Single-base substitutions resulting in SNPs or mutations arise through shared mechanisms, primarily single-base slip mispairing during DNA replication and CpG-mediated cytosine deamination (25-27).

EDN-1 presents three important SNPs, two in the promoter region [−974CNA (rs3087459) and−1394TNG (rs1800541)] and one in the coding region [Glu105Glu (rs5369)]. The two SNPs in the promoter region have been identified in the 5′-flanking

region of the EDN-1 gene and both SNPs have been associated with risk of developing myocardial infarction, essential left ventricular hypertrophy, asthma, and rheumatoid arthritis (28-31). The SNP located in the coding region has been associated with asthma and high blood pressure (30,31). On the other hand, the eNOS gene presents two relevant SNPs in the promoter region [−786TNC (rs2070744) and−1474 ANT (rs1800783)] and one in the exon 7 of the coding region (G894T). The SNPs in the promoter region have been associated with a significant reduction in the eNOS gene promoter activity, whereas the SNP in the coding region leads to an amino acid change from Glu to Asp (Glu298Asp) and has been associated with reduced basal NO production. These eNOS variants have been associated with an increased risk for coronary artery disease, heart failure, atherosclerosis, and myocardial infarction (24, 32-36).

1.4 Aim of the study

Considering the prominent role of ET-1 and eNOS as regulators of endothelial function, inflammatory processes, and vascular physiology, they seem to have a measurable influence on the development of the atherosclerotic plaque and contribute to or increase the occurrence of ACS. In this context, the objective of this study was to establish the role of EDN-1 and eNOS gene polymorphisms in the risk of developing ACS in a group of Mexicans patients.

2. METHODS

2.1 Study population

The clinical characteristics of the study population are reported in the Table 1. We included 218 Mexican patients with ACS (148 males, mean age of 60.4 ± 11.7) referred to the National Institute of Cardiology Ignacio Chávez. Of them, EDN-1-974CNA

(rs3087459), EDN-1-1394TNG (rs1800541), and EDN-1 Glu105Glu (rs5369) single nucleotide polymorphisms were genotyped using 5′ exonuclease TaqMan genotyping assays on an ABI Prism 7900 HT Fast Real time PCR System, according to manufacturer's instructions. Acute coronary syndromes were diagnosed on the basis of clinical history, physical examination with electrocardiography, chest radiography, echocardiography, and coronary angiography. ACS diagnosis was made according to the World Health Organization and the American Heart Associated (AHA)/American College of Cardiology (ACC) criteria (37). As control, 204 healthy unrelated individuals (83 males, mean age of 59.4 ±9.8) with neither symptoms nor previous diagnosis of cardiovascular problems. All included subjectswere ethnically matched, and we considered as Mexican Mestizos only those individuals who for three generations, including their own, had been born in Mexico. Clinical characteristics of study population are summarized in the Table 1.

2.3 DNA extraction

The DNA extraction method proposed by Lahiri and Numberger was used for DNA extraction (Lahiri and Numberger, 1991). Briefly, 500 µl of whole blood was mixed with 500 µl of TKM buffer (Tris–HCl pH 7.6 10 mM, KCl 10 mM, MgCl2 10 mM, EDTA 2 mM) in a microcentrifuge tube. To this, 1 ml of TKM + 2.5% Triton X-100 was added to disrupt the cell membrane without damaging nuclei. The tube was mixed by inversion (10 times) and then centrifuged at 1000 g for 8 min at 4 °C. The cytoplasmic supernatant was discarded and the nuclear pellet was resuspended in 1 ml of TKM by pipetting. Cells were again centrifuged at 1000 g for 8 min and washed with TKM once more. The final pellet was resuspended in 200 µl TKM, after which 15 µl of 10% SDS was added to lyse nuclei and mixed by inversion. Material was incubated for 5 min at 55 °C and 75 µl of

saturated NaCl solution was added. The tube was mixed by inversion and centrifuged at maximum speed (12,600 g) in an Eppendorf microcentrifuge for 5 min at room temperature. Supernatant was transferred to a fresh tube and 0.7 volume of isopropanol was added. The sample was mixed by inversion and centrifuged for 10 min at maximum speed in a microcentrifuge. Pellet was washed in cold 70% ethanol and permitted to air dry for 15 min. DNA material was resuspended in 100 µl TE at 65 °C for 1 h.

2.3 Determination of eNOS and EDN-1 genotypes

ENOS298, eNOS785, eNOS1474, EDN-1-974CNA (rs3087459), EDN-1-1394TNG (rs1800541), and EDN-1 Glu105Glu (rs5369) single nucleotide polymorphisms were genotyped using 5′ exonuclease TaqMan genotyping assays on an ABI Prism 7900 HT Fast Real time PCR System, according to manufacturer's instructions.

2.4 Statistical analysis

The distribution of continuous variables was assessed by visual inspection of frequency histograms and with the use of the Shapiro–Wilk test. Continuous variables were expressed as mean ± standard deviation (SD) or median with interquartile range, if they followed a normal or non-normal distribution, respectively. Continuous variables were compared with unpaired t-test or Mann–Whitney U-test, whereas categorical variables were compared using the chi square test or Fisher's exact test, as appropriate. Correlations between variables were performed using the Pearson test.

All variables were tested in an univariable model and then entered in the multivariable model if p-value was < 0.05. The software SPSS 17.0 (SPSS Italy, Florence, Italy) was used for statistical analyses.

3. RESULTS

3.1 Study population

Results of the study population are summarized in the Table 1. Patients with ACS were older than healthy subjects (<0.001). Moreover, they had more frequently family history (<0.001), smoking habitus (<0.001), hypertension (<0.001) and diabetes mellitus (<0.001) compared to control group. Yet, patients with ACS presented an enhances risk of cardiovascular disease.

3.2 Genetic profile of study groups

Results of genetic profile of study population are summarized in the Table 2. An increased frequency of EDN-1 Glu105Glu (rs5369) polymorphism was found in ACS patients compared to healthy subjects (p=0.004) (Figure 1). Within patients with ACS, patients with hypertension showed more frequently eNOS 1474AA polymorphism compared to those without hypertension (p=0.0022) (figure 2, panel A). Yet, obese patients presented more frequently eNOS 1474AA (p=0.001), eNOS-1474T/A (p=0.004) and eNOS786CC (p=0.011) polymorphisms than those without obesity (figure 2, panel B).

3.2 Predictors of ACS

Predictors of ACS are reported in the Table 3. At univariate analysis, predictors of ACS are age, male sex, hypertension, family history of CAD, high CV risk and EDN-1 Glu105Glu (rs5369) polymorphism. At multivariable analysis, age, male sex, family history of CAD, high CV risk and EDN-1 Glu105Glu (rs5369) polymorphism were independent predictors of ACS.

4. DISCUSSION

Our study shows that, in a Mexican population, 1) compared to healthy subjects, patients with ACS exhibit an increased frequency of EDN-1 Glu105Glu (rs5369) polymorphism; 2) within patients with ACS, patients with hypertension showed more frequently eNOS 1474AA polymorphism compared to those without hypertension; 3) obese patients presented more frequently eNOS 1474AA, eNOS-1474T/A and eNOS786CC polymorphisms than those without obesity; 4) age, male sex, family history of CAD, high CV risk and EDN-1 Glu105Glu (rs5369) polymorphism were independent predictors of ACS.

Susceptibility to CAD is claimed to be 40% to 60% inherited, but until recently genetic risk factors predisposing to CAD have been elusive. Routine genetic screening is unlikely until management is improved by genetic testing. Risk variants should provide pathophysiological insights and targets for novel therapy. While risk variants are less potent predictors of CAD, compared with biomarkers, they have the advantage of not changing in one's lifetime and are unaffected by diet, sex, age, or medication. The most important genes responsible for vascular physiology and severity of coronary diseases, are represented by ET-1 and eNOS. (10).

ET-1 as a potent vasoconstrictor and modulator of vasomotortone and vascular remodelling (11,12). Yet, ET-1 has a pro-atherogenic activity and mediates smooth muscle cell proliferation via ET-1 receptors. Experimental model showed as ET-1 and ET receptors are upregulated in both human and experimental animal atherosclerotic lesions. This notion has been further supported by a recent finding that administration of ET receptor antagonists resulted in a significant reduction of atherosclerosis in apoE-KO mice. Moreover, it acts as a chemoattractant for monocytes, and induces platelet

aggregation and expression of adhesion molecules (14-16); 4) finally, it stimulates superoxide production, NF-κB expression, and atherosclerotic lesion formation, and inhibits eNOS (17). On the other hand, eNOS is considered a protective enzyme, not only for its role in NO synthesis but also because its inhibition is associated with the production of superoxide (18,19). Vascular NO· dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in smooth muscle cells. NO· released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. NO· also can inhibit leukocyte adhesion to the vessel wall either by interfering with the ability of the leukocyte adhesion molecule CD11/CD18 to form an adhesive bond with the endothelial cell surface or by suppressing CD11/CD18 expression on leukocytes. White cell adherence is an early event in the development of atherosclerosis; therefore, NO· may protect against the onset of atherogenesis. Furthermore, NO· has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells. The inhibition of platelet aggregation and adhesion protects smooth muscle from exposure to platelet-derived growth factor(s). Therefore, NO· also prevents a later step in atherogenesis, fibrous plaque formation. Based on the combination of those effects, endothelial NO· probably represents the most important antiatherogenic defense principle in the vasculature (21). For these reasons, several studies have assessed the correlation between endothelial expression levels of ET-1 and eNOS with the instability of the atherosclerotic plaque. These genetic factors could act with the classical risk factors (e.g. hypertension, obesity, male sex, age, etc.) in order to cause a cardiovascular event.

5. LIMITATIONS OF THE STUDY

The mayor limitation of the study is the small sample size. Indeed, further larger studies are required to confirm these results. Moreover, the lack of a follow-up does not allow to get informations about the prognostic implication of these polymorphisms.

6. CONCLUSION

Resulting data suggest that EDN-1 Glu105Glu (rs5369) polymorphism could be involved in the risk of developing ACS in Mexican in patients. Risk variants should provide pathophysiological insights and targets for novel therapy.

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8. TABLES AND FIGURES

Table 1 Clinical characteristics of study groups

	Case (n 218)	Control (n 204)	p
Age, yrs, mean±SD	60.39±11.07	58.9±9.85	<0.001
Male , n (%)	148 (67)	83 (40)	<0.001
Smoking, n (%)	130 (59)	27 (13)	<0.001
Diabetes Mellitus, n (%)	81 (37)	26 (12)	<0.001
Hypertension, n (%)	131 (60)	85 (41)	<0.001
Dislipidemia, n (%)	133 (61)	111 (54)	0.102
Family history of CAD, n (%)	118 (25)	14 (6)	<0.001
Obesity, n (%)	47 (21)	33 (16)	0.277
CV Risk grade, n(%) Mild Moderate high	 38 (17) 56 (25) 124 (56)	 86 () 61 (30) 57 (28)	<0.001
TC, mg/dl, median (IR)	169.0 (138.0-197.0)	175.0 (150-195)	0.220
HDL, mg/dl, median (IR)	40.0 (34.0-47.65)	41.8 (34.0-48.0)	0.360
LDL, mg/dl, median (IR)	93.0 (72-0-117.0)	98.0 (75.2-118.5)	0.269
TG, mg/dl, median (IR)	144.5 (108.6-204.5)	140.5 (104.0-201.5)	0.441

Coronary artery disease, CAD; CV, cardiovascular risk factors; interquartile range, IR; standard deviation, SD; total cholesterol, TC; LDL, HD

Table 2 Genetic profile of study groups

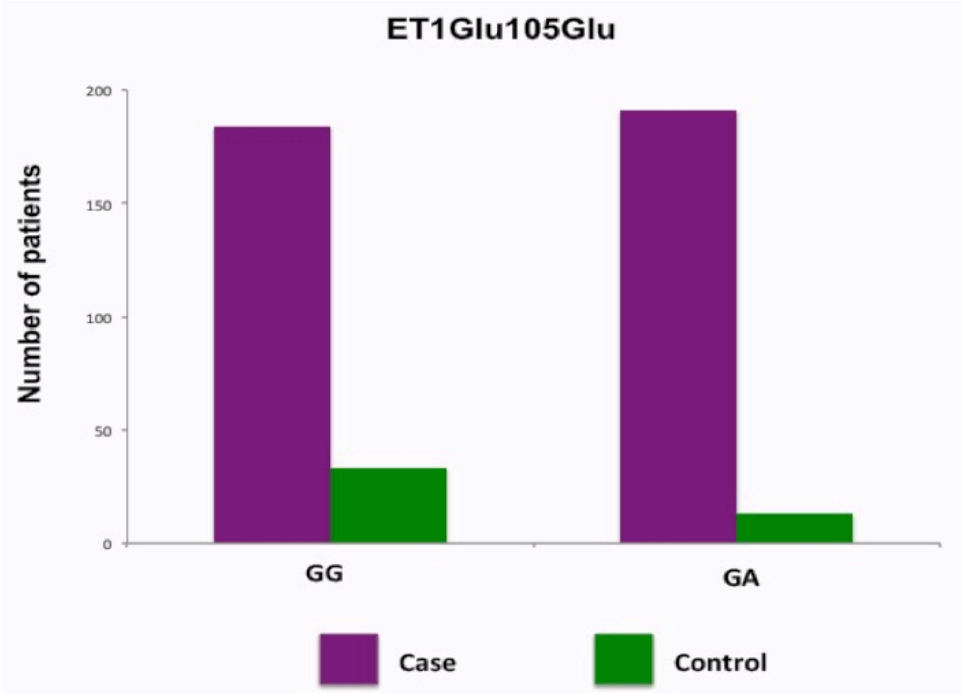
	Case (n 218)	**Control (n 204)**	**p**
eNOS2980, n (%)			0.441
GG	138	140	
GT	66	55	
TT	14	9	
eNOS785, n (%)			0.121
TT	123	135	
TC	81	59	
CC	14	10	
eNOS1474, n (%)			0.058
TT	119	130	
TA	88	60	
AA	11	14	
ET1974, n (%)			0.551
AC	41	34	
AA	176	170	
ET1394, n (%)			0.432
TT	171	170	
TG	46	33	
GG	1	1	
ET1Glu105Glu, n (%)			**0.004**
GG	**184**	**191**	
GA	**33**	**13**	

Table 3 Independent predictors of ACS.

	Univariable regression model		**Multivariable regression model**	
	HR (CI 95%)	**p**	**OR (CI 95%)**	**p**
Age	0.010 (0.005-0.014)	<0.001	0.127.(-0.001-0.008)	0.127
Male	0.274 (0.182-0.367)	<0.001	0.171 (0.086-0.257)	<0.001
Smoking	-0.065 (-0.132-0.001)	0.055		
Hypertension	0.018 (0.085-0.275)	<0.001	0.080 (-0.002-0.163)	0.055
Diabetes Mellitus	0.322 (0.216-0.428)	0.054		
Family history of CAD	0.549 (0..460-0.638)	<0.001	0.459 (0.410-0.580)	<0.001
CV risk grade	0.190 (0.136-0.244)	<0.001	0.121 (0.011-0.132)	0.020
Dislipidemia	0.068.(-0.029-0.164)	0.171		
ET1Glu105Glu	0.227.(0.074-0.379)	0.004	0.129 (0.082-0.329)	0.001

Coronary artery disease, CAD; confidence of interval, CI; CV, cardiovascular risk factors; Hazarrd ratio, HR. Odds ratio, OR.

Figure 1



9. LEGENDS

Figure 1.

Distribution of ETGlu105Glu polymorphism in case and control groups.