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Analysis of polymorphisms and haplotypes in genes associated with vascular tone, hypertension and oxidative stress in Mexican-Mestizo women with severe preeclampsia

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ABSTRACT

Objective: Several studies have reported the association of genes related to vascular tone, hypertension, oxidative stress and preeclampsia. We investigated the possible association among three polymorphisms in *eNOS* (as well their haplotypes): one of *MTHFR*, one of *GSTP1* and one of *AGT*, with severe preeclampsia in Mexican-Mestizo women.

Methods: Two hundred thirty women with severe preeclampsia and 350 control subjects were genotyped; for rs2070744 and rs1799983 of *eNOS*, rs1801133 of *MTHFR*, rs1695 of *GSTP1* and rs699 of *AGT* we used real-time PCR allelic discrimination and for VNTR of *eNOS*, PCR. Allele frequency differences were assessed by χ^2. Logistic regression was used to test for associations and for haplotype frequencies using Haploview 4.2.

Results: Genotypic and allelic distribution of the polymorphisms was similar between cases and controls; likewise, haplotype frequencies of the three polymorphisms of *eNOS* did not differ significantly.

Conclusions: To our knowledge, this is the first time that these polymorphisms have been analyzed together and exclusively in women with severe preeclampsia. However, we did not find an association between polymorphisms of *eNOS*, *MTHFR*, *GSTP1* and *AGT* with severe preeclampsia in our population. Additionally, we observed differences in the distribution of the alleles and genotypes of these polymorphisms in our population in comparison to those described in other ethnic groups.

Introduction

Preeclampsia is a common multifactorial disease and one of the major causes of fetal and maternal morbidity and mortality in pregnancy. The severe form of this syndrome has the highest complication rates [1,2].

The mechanisms causing preeclampsia are still unclear; however, a genetic susceptibility to this syndrome has been established. Several studies have reported associations of preeclampsia and genes related to vascular tone, hypertension and oxidative stress-related genes. Among these genes are *endothelial nitric oxide synthase* (*eNOS*) (MIM 163729), *5,10 methylenetetrahydrofolate reductase* (*MTHFR*) (MIM 607093), *angiotensinogen* (*AGT*) (MIM 106150), and *glutathione S-transferase P1* (*GSTP1*) (MIM 134660).

A reduced nitric oxide (NO) formation has been implicated in the pathogenesis of this syndrome [3,4]. The endothelial NO synthase (*e*NOS) is the enzyme that generates NO in blood vessels and regulates vascular function, as well as playing a protective role on endothelial cells [5,6]. Three polymorphisms in *eNOS* and their haplotypes have been associated with preeclampsia: the −786T→C (rs2070744), a variable number of *tandem* repeats (VNTR) in intron 4, and

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G-894T→Glu298Asp (rs1799983) [7,8]. Furthermore, hyperhomocysteinemia has also been described as a risk factor for preeclampsia õbecause it may lead to endothelial dysfunction in affected women [9,10]. The polymorphism C-677T→A222V in *MTHFR* (rs1801133) encoding a thermally unstable protein with reduced activity and resulting in elevated plasma homocystine levels has been associated with preeclampsia [11,12].

Moreover, the renin–angiotensin system plays an important role in blood pressure regulation through its components like angiotensinogen (AGT), which plays crucial roles in the regulation of blood pressure. C-704T→Met235Thr in *AGT* has been associated with an increased risk of development of preeclampsia. Homozygous *TT* individuals have increased plasma levels of angiotensinogen [13–16]. In addition, oxidative stress is thought to also play an important role in the pathophysiology of this syndrome. Glutathione S-transferases (GSTs) protect cells against the cytotoxic effects of reactive compounds [17,18]. Similarly, the variant A-313G→Ile105Val (rs1695), which reduces the catalytic activity of the enzyme, has been associated with this syndrome [12,19]. It has been suggested that reduced levels of GSTP1 in preeclampsia may be an indicator of decreased capacity of the GST detoxification system and possibly may cause a prolonged exposure to reactive by-products, which may contribute to maternal endothelial dysfunction [18,19]. However, the results of studies seeking associations of these polymorphisms in *eNOS*, *AGT*, *MTHFR* and in *GSTP1* with preeclampsia have not always been consistent in different population analyses [7,20–22]. Due to the fact that important ethnic differences have been observed in risk of preeclampsia [16,23,24] and the Mexican-Mestizo population is comprised of a mixture of Europeans and Africans with native Indian subjects [25], the principal aim of this study was to analyze the possible association among three polymorphisms in *eNOS* (as well their haplotypes) and one polymorphism in *AGT*, one in *MTHFR* and one in *GSTP1* genes with the presence of severe preeclampsia in Mexican-Mestizo women.

Subjects and methods

Subjects

The study was approved by the Institute's Human Research Committees. Informed consent was obtained from all patients and controls before participation in the study. A case–control study was performed. Two hundred thirty women with severe preeclampsia and 352 pregnant women without preeclampsia (controls) were analyzed; all were of Mexican-Mestizo ethnic origin. Only women born in México and with a Spanish-derived last name along with a family of Mexican ancestors dating back to the third generation were considered Mexican-Mestizo.

A research physician administered a questionnaire to all women to define the potential risk factors for preeclampsia such as maternal age, parity, gestational age at delivery, smoking status, alcohol intake, family history of preeclampsia, ethnicity, and socioeconomic status. Women with chronic hypertension, chronic nephropathy, diabetes mellitus, cardiovascular and other chronic diseases were excluded from the study.

Women admitted with a diagnosis of preeclampsia and who agreed to participate in the study were recruited and selected consecutively. Preeclamptic women were recruited from the Clinic of Hypertensive Diseases of Pregnancy, Unidad Médica de Alta Especialidad, Hospital de Ginecología y Obstetricia "Luis Castelazo Ayala," Instituto Mexicano del Seguro Social. Control subjects were women of the same origin recruited from the Hospital Regional "Adolfo López Mateos," Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado.

In all cases, a physician or obstetrical nurse measured blood pressure with subjects in a seated position using the auscultatory method with a mercury sphygmomanometer. Korotkoff phase V was generally used for defining diastolic blood pressure. Severe preeclampsia was defined as the development of hypertension and proteinuria (>5 g urinary protein in 24 h) in women with no baseline proteinuria. Hypertension was defined as systolic blood pressure >160 mm Hg and/or diastolic blood pressure >110 mm Hg measured on two consecutive occasions at least 24 h apart [26].

The control group was comprised of women with uncomplicated pregnancy admitted for natural childbirth or Caesarean section, with normal pregnancy duration, blood pressure <120/80 mm Hg and without proteinuria.

Genotyping

Peripheral blood samples were obtained from all women, and genomic DNA was purified using the salting out procedure as described by Miller et al. [27]. The following three polymorphisms in *eNOS* gene were studied: −786T→C in the 5′-flanking region (rs2070744), the VNTR (27 bp-repeat) polymorphism in intron 4 and the G-894T→Glu298Asp polymorphism in exon 7 (rs1799983), the C-677T→Ala222Val (rs1801133) in *MTHFR*, the C-704T→Met235Thr (rs699) in *AGT* and the A-313G→Ile105Val (rs1695) in *GSTP1*. Real-time PCR allelic discrimination TaqMan assay (AB) was used for genotyping −786T→C and G-894T→Glu298Asp of *eNOS*, C-677T→Ala222Val of *MTHFR*, C-704T→Met235Thr of *AGT* and A-313G→Ile105Val of *GSTP1*. All PCR reactions contained 10 ng of DNA, 5.0 μL TaqMan Universal Master Mix (AB) (2×), 0.25 μL primers and probes (10×) and water for a final volume of 10 μL including the appropriate negative controls in all assays.

In the case of the four polymorphisms described above, the assay used probes and primers designed by Applied Biosystems (Foster City, CA) assay-on-demand services: for Glu298Asp, assay ID: C___3219460_20; for Ala222Val of *MTHFR*, assay ID: C___1202883_20; for Met235Thr of *AGT*, assay ID: C___1985481_20 and for Ile105Val of *GSTP1*, assay ID: C___3237198_20. For the −786T→C polymorphism of *eNOS*, the probe and primers were designed by our group. Primer sequences were as follows: forward 5′-ACCAGGGCATCAAGCTCTTC-3′ and reverse 3′-GCAGGTCAGCAGAGAGACTAG-3′. The probe for each allele is as follows: wild-type FAM 5′-AGGGTCAGCCAGCCAG-3′ and mutant VIC 5′-AGGGTCAGCCGGCCAG-3′. Real-time PCR was performed on an ABI Prism 7500 Fast (Applied Biosystems). Conditions for Glu298Asp of *eNOS*, Ala222Val of *MTHFR*, Met235Thr of *AGT*, and Ile105Val of *GSTP1* were 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of amplification (92 °C for 15 s and 60 °C for 1.30 min).

For the −786T→C polymorphism of *eNOS*, conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of amplification (92 °C for 15 s and 60 °C for 1 min). For each cycle, the software determined the fluorescent signal from the VIC or FAM-labeled probe (Applied Biosystems). Allelic discrimination was performed using specific primers and probes for each allele.

Genotypes for VNTR in intron-4 of *eNOS* were determined by PCR. DNA was amplified by the polymerase chain reaction (PCR) in 25 μL of reaction mixture containing 200 ng of genomic DNA, 0.2 mM dNTPs, 2.0 U of *Platinum Taq* DNA polymerase (Invitrogen, Life Technologies Corporation, São Paulo, Brazil), and 0.2 μM of each specific set of intron 4 of the *eNOS* primers (primer sequences have been described previously) [28]. Thirty cycles of PCR amplifications were performed in a Thermal Cycler (Multigene II, Labnet International Inc., Woodbridge, NJ, USA). Except for the last, all cycles were 1 min at 96 °C, 1 min at 59 °C, and 1 min at 72 °C. In the final cycle the annealing temperature was at 72 °C for 5 min. After amplification, PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide to verify the correct size of the expected fragments, the negative control in the PCR having all reagents except DNA. Three alleles were obtained when this region was amplified: "*eNOS*a," which was 234 base-pair (bp) long and consisted of one