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eNOS gene Glu298Asp and 4b/a polymorphisms are associated with renal function parameters in Mexican patients with Fabry disease

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ABSTRACT. Fabry disease (FD) is an inherited X-linked lysosomal disease that causes renal failure in a high percentage of affected individuals. The *eNOS* gene encodes for endothelial nitric oxide synthase, which plays an important role in glomerular hemodynamics. This gene has two main polymorphisms (Glu298Asp and 4b/a) that have been studied in the context of many different diseases, including those involving cardiovascular and renal alterations. Considering the

lack of information regarding *eNOS* variants and FD, we investigated whether there were associations between *eNOS* genetic variants and renal function parameters in Mexican patients with FD and renal impairment. In total, 15 FD patients with renal alterations were included in the present study, and associations between *eNOS* polymorphisms and renal function parameters (urea, creatinine, and GFR) were evaluated. The Asp298 and 4a alleles of the *eNOS* gene were found to be significantly associated with increased levels of urea and creatinine, and a decreased glomerular filtration rate in FD patients, and this association behaved in a co-dominant fashion. Our results coincide with previous reports showing an association between these polymorphisms and kidney disease, and along with other studies regarding their role in the nitric oxide pathway, suggest that these variants affect the severity of nephropathy in patients with FD.

Key words: *eNOS* polymorphisms; Glu298Asp; 4b/a (VNTR); Fabry disease; Renal function parameters; Kidney disease

INTRODUCTION

Fabry disease (FD) is an inherited X-linked lysosomal storage disease caused by mutations in the α-galactosidase (*GLA*) gene located at chromosome region Xq22.1. The lack of α-galactosidase A (α-GLA-A) enzyme activity in FD produces an accumulation of neutral glycosphingolipids, particularly globotriaosylceramide, in multiple organs and organ systems including the heart, skin, eyes, and kidneys, as well as the gastrointestinal, auditory, and nervous systems, and this occurs mainly in capillary endothelial cells (Ramos-Kuri et al., 2014). One of the most severe manifestations of FD is renal failure, which is one of the main causes of death in these patients (Waldek and Feriozzi, 2014).

The *eNOS* gene encodes for endothelial nitric oxide synthase (NOS), which is located at chromosome region 7q35-36. NOS has numerous functions in the kidney including the control of renal and glomerular hemodynamics, and promotes natriuresis and diuresis along with renal adaptation to dietary salt intake (Dellamea et al., 2014). Two main polymorphisms of the *eNOS* gene have been studied in different diseases, and these are the G894T or Glu298Asp (rs1799983) and the 27-bp repeat in intron 4 (VNTR) 4b/a variants, which have been found to be associated with diabetic nephropathy in a meta-analysis that included more than 12 studies (Dellamea et al., 2014).

In regards to these two *eNOS* polymorphisms in FD, two studies have been performed. Specifically, Heltianu et al. (2002) showed a significant increase of Asp298 allele frequency in patients with FD compared to that of controls, and also that the Asp298 and 4a alleles were significantly associated with renal failure in 37 patients with FD (Heltianu et al., 2005). However, these studies did not investigate associations between these genetic variants and renal function parameters. Therefore, in the current study, we investigated potential associations between the presence of these genetic variants and renal function parameters in patients with FD and renal failure in order to determine if these variants exert some effect on the severity of renal disease.

MATERIAL AND METHODS

Patients

Fifteen patients with FD residing in western Mexico were included in this study, two of which were brothers and the other 13 were unrelated individuals. All FD patients were under the medical care of nephrology services at third-level attention hospitals. The criteria for inclusion were FD confirmed by the absence or diminution of α-GLA-A activity, renal affection with an increase in urea and creatinine levels above normal limits, and no other associated pathology. All investigations were conducted according to the principles expressed in the Declaration of Helsinki, and all included patients provided written informed consent prior to enrollment in the study. This study was approved by the Ethics Committee of the Instituto Mexicano del Seguro Social (IMSS) (approval No. R-2011-785-052).

Polymorphism detection

DNA was extracted according to methods described by Miller et al. (1988). For detection of the Glu298Asp polymorphism, the following primers were used: forward 5'-AAGGCAGGAGACAGTGGATGGA-3' and reverse 5'-CCCAGTCAATCC CTTTGGTGCTCA-3'. The 248-bp amplified fragment was then digested with the *Ban*II restriction enzyme, which produces fragments of 163 and 85 bp in the case of the Glu298 allele, whereas the Asp298 allele results in a band of 248 bp. For detection of the 27-bp repeat in intron 4 (VNTR) 4b/a, the following primers were used: forward 5'-AGGCCCTATGGTAGTGCCTT-3' and reverse 5'-TCTCTTAGTGCTGTGGTCAC-3'. This amplified a fragment of 421 bp when the insertion allele (4b) was present, and a 394-bp fragment when the deletion allele (4a) was present. The amplified products were visualized on 6-8% acrylamide gels.

Renal function parameters

For all patients, data on urea and creatinine concentrations (reported herein as mg/dL), and glomerular filtration rate (GFR) (reported herein as mL x min^{-1}·$(1.73\ m^2)^{-1}$) were obtained from clinical records during their first nephrology service.

Statistical analysis

Quantitative variables are reported as means ± standard deviation (SD), and qualitative variables are reported as frequencies. In order to compare the renal function parameters among the different genotypes, the Kruskal-Wallis test was conducted to compare more than 2 groups and Mann-Whitney U-tests were used to compare two groups. All statistical analyses were performed using the SPSS v.10.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

All of the patients were males, and presented an average age of 23.87 ± 2.90 years and a range from18 to 29 years. There were no significant correlations between renal function parameters and age.

Glu298Asp polymorphism

Three patients were homozygous Glu/Glu, two patients were homozygous Asp/Asp, and ten patients were heterozygous Glu/Asp. The allele frequency for the Glu298 allele was 53 and 47% for the Asp298 allele. Additionally, the genotype distribution was within Hardy-Weinberg equilibrium.

In the association analysis, there was an observed significant difference between individuals with the Asp/Asp genotype and those with the Glu/Asp genotype in regards to their urea (120.5 ± 30.41 *vs* 73.40 ± 11.19) and creatinine (9.5 ± 2.68 *vs* 4.62 ± 1.46) concentrations, as well as GFR (11.30 ± 9.48 *vs* 35.30 ± 14.10), P = 0.030 for all the three variables. Significant differences were also observed between the Glu/Glu and Glu/Asp genotypes in regards to urea (54.33 ± 5.13 *vs* 73.40 ± 11.19, P = 0.014) and creatinine (2.63 ± 0.115 *vs* 4.62 ± 1.46, P = 0.049) concentrations, in addition to GFR (61.67 ± 18.82 *vs* 35.30 ± 14.10, P = 0.049).

VNTR 4b/a (27-bp) polymorphism

Twelve patients presented the 4b/b genotype and 3 patients the 4b/a genotype. The allele frequency for the 4b allele was 90%, and was 10% for the 4a allele. The genotype distribution was within Hardy-Weinberg equilibrium. In the association analysis, we observed significant differences between the 4b/b and 4b/a genotypes in regards to urea (66.92 ± 11.04 *vs* 111.67 ± 26.39, P = 0.004), creatinine (3.91 ± 1.31 *vs* 8.70 ± 2.35, P = 0.004), and GFR (43.17 ± 18.10 *vs* 14.20 ± 8.37, P = 0.004).

DISCUSSION

To our knowledge, this is the first study to investigate associations between the Glu298Asp and 4b/a *eNOS* genetic variants and renal function parameters in FD patients with renal failure. With respect to the functional effects of the Glu298Asp polymorphism, it has been shown that the Asp298 allele undergoes selective proteolysis in native cells and tissues that could reduce the steady-state level of active eNOS (Tesauro et al., 2000; Hingorani, 2003). However, these observations were not confirmed in another study (McDonald et al., 2004), and it was shown that the enzymatic activity was identical for both alleles (Hingorani, 2003; McDonald et al., 2004). Nevertheless, this polymorphism has been shown to be associated with a number of diseases/conditions including diabetic nephropathy (Dellamea et al., 2014), preeclampsia (Qi et al., 2013), myocardial infarction (Hingorani et al., 1999), and with renal failure in FD patients (Heltianu et al., 2005).

In agreement with previously results, we also found that there were associations between the Asp298 allele and increased urea, creatinine, and GFR. These associations were compatible with a co-dominant effect, resulting in higher urea and creatinine concentrations and lower GFR in the Asp/Asp homozygotes than those of the Glu/Asp heterozygotes, which were in turn higher and lower, respectively, than those in the Glu/Glu homozygotes. These associations were not biased by age given that no correlation between the renal function parameters and age was found. However, the values of these parameters were taken from the first visit to the nephrology service, and thus the precise timing of the renal alterations remains unknown.

Interestingly, we observed that the frequency of the Asp298 polymorphic allele (47%) was higher than in most populations, including those in Sweden, Brazil, Asian countries

(Japan, South Korea, Western Iran, and Asian Indian), and African countries (Egypt and Tunisia), whose frequencies range from 5 to 34% (Dellamea et al., 2014).

The 27-bp repeat in intron 4 (VNTR) 4b/a has been shown to reduce the expression of *eNOS* mRNA and protein levels in transfected endothelial cells (Zhang et al., 2005) by altering histone acetylation and DNA methylation in regions adjacent to the 27-bp repeat element and core promoter (Zhang et al., 2008). While some studies have reported that there is an association between the wild-type 4b allele and diabetic nephropathy (Dellamea et al., 2014), hypertension, and systemic lupus erythematosus (AlFadhli, 2013), other studies have found an association between the 4a allele and coronary artery disease (Yang et al., 2014), cancer (Zhang et al., 2014) and systemic lupus erythematosus (Sandoughi et al., 2014). Here, we observed an association between the 4b/a genotype and increased levels of urea and creatinine and lower GFR when compared with those of the 4b/b genotype.

The *eNOS* polymorphic allele frequencies observed herein (10%) were similar to those reported in other countries including the United States, Japan, China, Asian India, Brazil, and the United Kingdom, which range from 7.3 to 14.8%, and were somewhat lower than those reported in Sweden, Poland, Germany, Tunisia, and Egypt, which range from 16.5 to 23.4% (Dellamea et al., 2014).

In conclusion, we found that there was an association between the Asp298 and 4a alleles of the *eNOS* gene and reduced renal function in Mexican FD patients, suggesting that these variants may play a role in the severity of nephropathy in these patients. However, future studies that include larger sample sizes and more clinical variables are needed to elucidate the roles that different genetic variants play in the renal affection of FD.

Conflicts of interest

The authors declare no conflict of interest.

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