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PRELIMINARY REPORT

Association of the Vascular Endothelial Growth Factor Gene Polymorphism +936 C/T with Diabetic Neuropathy in Mexican Patients with Type 2 Diabetes Mellitus

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Background. Peripheral neuropathy is one of the most common late complications of diabetes. Vascular endothelial growth factor (VEGF) gene polymorphisms have been associated with the development of peripheral neuropathy in different populations of patients with type 2 diabetes mellitus (DM2).

Objective. To analyze the prevalence of the +936 C/T *VEGF* gene polymorphism among patients with DM2 with and without peripheral neuropathy.

Study design and methodology. 218 unrelated DM2 patients, 90 with and 128 without peripheral neuropathy were genotyped for the +936 C/T *VEGF* gene polymorphism using PCR amplification followed by restriction length polymorphism analysis.

Results. The CC homozygous VEGF+936 C/T (rs3025039) was the predominant genotype in DM2 patients with peripheral neuropathy, whereas the predominant genotype in patients without neuropathy was the heterozygous C/T. No statistical association was found between genotype distribution and the presence of neuropathy ($p = 0.063$). The distribution of the genotypes according to the dominant (CC vs. CT + TT) and recessive (TT vs. CT + CC) models showed that the homozygous CC and TT genotypes, respectively, are not risk factors for neuropathy. The CT genotype conferred a protective effect as seen in the over-dominant model (CT vs. CC + TT) (OR = 0.52; 95% CI = 0.300–0.90; $p = 0.019$).

Conclusion. We conclude that the VEGF+936 C/T (rs3025039) gene polymorphisms are related to peripheral neuropathy in Mexican DM2 patients, with the heterozygous genotype potentially conferring a protective effect.

Key Words: Vascular endothelial growth factor, Diabetic peripheral neuropathy, Polymorphism.

Introduction

Microvascular complications in diabetes mellitus include retinopathy, nephropathy and neuropathy (1,2). At least 50% of patients with type 2 diabetes mellitus (DM2) suffer diabetic peripheral neuropathy (DPN), which is the most common form of neuropathy and a major cause of limb amputations (2,3).

Many of the biological and biochemical changes that occur early in vascular and neuronal cells are induced by hyperglycemia (1,3). Prolonged hyperglycemia results in the induction o the sorbitol pathway, hypoxia and oxidative stress, as well as an increment in advanced glycosylation end products. Those mechanisms trigger the production of

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vascular endothelial growth factor (VEGF). VEGF is an endothelial cell-specific mitogen with two different roles in the pathogenesis of DPN. First, induced by hypoxia, VEGF increases vascular permeability and causes inflammation; and second, VEGF acts as a protective agent by inducing nerve regeneration (4−6).

A possible role of polymorphisms of the *VEGF* gene in microvascular complications has been suggested (1,4−6). VEGF+936 C/T (rs3025039) polymorphism is located in the 3′ untranslated region and was noted by others to be related to plasma VEGF levels (7,8).

The purpose of the present study was to evaluate the association of the +936C/T (rs3025039) polymorphism in the *VEGF* gene with diabetic neuropathy in DM2 patients.

Materials and Methods

Subjects

A comparative case-control study was performed to analyze the clinical characteristics of DPN and to explore the genetic association between DPN and polymorphic variants of the VEGF gene. The study population included 218 unrelated DM2 Mexican patients consecutively enrolled from the Mexican Social Security Institute hospital (Saltillo, Coahuila, Mexico). The DM2 patients were part of an integral diabetes mellitus program offered in a hospital in which the patients received medical, nutritional and psychological care. From a total of 400 patients included in the hospital's diabetes program, 182 patients were excluded according to the following criteria: patients who did not agree to participate in the study; those with incomplete biochemical analyses or who did not attend a medical consultation; patients with DM1 or with neuropathy of non-diabetic origin; patients with peripheral vascular disease of the pelvic limbs; and those with renal failure.

Diabetes was defined according to the guidelines set by the American Diabetes Association (9). The present study focused only on the neuropathic processes and not on vascular ones. The 10 g monofilament-test and tuning fork test were used for assessing foot sensitivity (10). The diagnosis of DPN was made by having abnormal monofilament or abnormal tuning fork test results. A DPN diagnosis was made after abnormal results were found on either the monofilament or tuning fork test.

The minimum number of DPN patients required to achieve a statistical power of 80% was calculated based on the prevalence of DPN in the studied population and using the formula based on the estimation of proportion.

The study protocol was performed in accordance with the ethical standards approved by the Institutional Review Board of the hospital (FM001-15). Informed consent was obtained from all subjects enrolled following an explanation of the nature of the study.

Clinical Data Collection and Study Group Classification

DM2 patients were classified into two groups, patients with and without peripheral neuropathy (DPN group and non-DPN group, respectively). DNP was diagnosed in a masked manner by independent specialized physicians through the sensation of vibration with a 128 Hz tuning fork (Riester C128, Germany) applied to different sites on the patients' legs and feet. The vibration fork was either felt or not, so the patients' responses were ''yes'' or ''no.'' The vibration perception was evaluated six times per foot. The results obtained from each foot were averaged separately, and patients who had more than three incorrect answers were reported as vulnerable to ulcers. A second test was conducted using a 10 g monofilament (Bailey W-CH533, Rhitmey, UK) applied to a non-callused site. It was repeated six times on both feet, over the first, third and fifth metatarsal head of the dorsal side and on the plantar side of the foot. The 10 g filament was applied to each site, and the patient was asked if he or she felt the filament. Four correct answers out of six applications for each side were considered normal.

The following clinical and biochemical characteristics were analyzed for all patients: age (in years), gender, duration of diabetes (in years), systolic and diastolic pressure (mmHg), BMI (weight/height2), fasting plasma glucose (mg/dL), cholesterol (mg/dL), triglycerides (mg/dL), and HbA_{1c} (%). Peripheral blood was obtained from all patients following a 12 h fast for biochemical analysis. Biochemical assays were performed on the Architect Plus C4000 (Abbott Laboratories, Chicago, IL, USA).

DNA Extraction

Peripheral venous blood samples (5 mL) were obtained from the DPN and non-DNP patients and were collected in EDTA tubes (BD Vacutainer®; Becton, Dickinson Co., Franklin Lakes, NJ, USA). The samples were centrifuged, and the buffy coat was processed for high-molecular weight genomic DNA isolation by the salting-out method (11). The samples were centrifuged using a refrigerated centrifuge (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the buffy coat was processed for high-molecular weight genomic DNA isolation by phenol-chloroform extraction, precipitated in ethanol, resuspended in Tris-EDTA (pH 7.8; Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), at a final concentration of 0.1−1.0 μg/μL, and stored at −20°C until analysis.

Genotyping for VEGF +936C/T (rs3025039) Polymorphism

Genotype and allele frequencies for the VEGF +936C/T polymorphism were performed by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) using an MJ Mini PTC1148 thermal cycler (Bio-Rad, Hercules, CA, USA) followed by NlaIII (New

England BioLabs [NEB], Ipswich, MA, USA). Restriction enzyme analysis was carried out as reported by Candan F, et al. (12). In brief, 250 ng genomic DNA, 0.5 mmol primers (forward 5′-AGGGTTCGGGAACCAGATC-3′ and reverse, 5′CTCGGTGATTTAGCAGCAAG-3′), 0.2 mmol deoxyribonucleotide triphosphates (Invitrogen Life Technologies, Carlsbad, CA, USA), 1.5 mmol MgCl2 and 2.5 units of Taq DNA polymerase (Invitrogen Life Technologies) were included in the reaction mixture. The 260 bp segment of the 3′-UTR region of VEGF encompassing the NlaIII site was amplified from genomic DNA in a final volume of 20 μL using the following PCR program: 95°C/30 s; 57°C/30 s; 72°C/30 s; 30 cycles.

The 260 bp amplicons (~1 μg) were digested overnight with NlaIII at 37°C and analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide, and visualized in a UVP model 2UV High Performance Trans illuminator (Upland, CA, USA). 260 bp band was observed in DM2 subjects who did not carry VEGF-936T polymorphism in both alleles (normal, VEGF-936C/C); 260-, 210- and 50 bp bands were observed in DM2 heterozygote subjects (VEGF-936C/T); and 210- and 50 bp bands were observed in DM2 subjects carrying VEGF-936T polymorphism in both of the alleles (homozygote VEGF-936T/T).

Statistical Analysis

All data were analyzed using SPSS software version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). Quantitative variables were expressed as means ± standard deviation and the qualitative values as percentages. Association between numerical variables with genotype and DPN were evaluated using *t*-test if the data showed normal distribution and Mann-Whitney *U* test if not. Association between polymorphism of VEGF gene 936 C/T and DPN was determined using χ^2 test. We estimated the recessive model, dominant model and over-dominant model for genotype comparison (13). Genotype distribution was assessed by Hardy-Weinberg equilibrium, using a goodness-of-fit test. A $p < 0.05$ were considered significant.

Results

Clinical Characteristics of the Study Population

The analyzed population comprised 218 clinically diagnosed DM2 patients. A prevalence of DPN of 41% ($n = 90$) was found in this DM2 study population. The rest of the DM2 patients (59%, $n = 128$) showed no signs of this microvascular complication (non-DPN group).

The DPN group patients had a mean age of 60.57 ± 10.26 years (range of 35−78 years), 63 (70%) were female and 27 (30%) male. The years of evolution of the DM2 was 12.39 ± 5.91, and the BMI had a mean of 30.53 ± 4.45. The non-DPN group patients had a mean age of 55.37 ± 11.23 (range of 30−82 years), 96 were female (75%) and 32 males (25%). The average years of evolution of the DM2 was 8.02 ± 5.87, and the BMI had a mean of 29.77 ± 4.29.

A comparison of averages of age, gender, years of evolution of diabetes and BMI was made between non-DPN and DPN groups. A significant difference was found with the mean age of the patients ($p = 0.001$) and the mean years of evolution of DM2 ($p = 9.05 \times 10^{-6}$) between both groups of patients. The χ^2 analysis with cut-off points between the DPN and non-DPN patients showed a significant association of age (>60 years) ($X^2 = 14.919$; 95% CI = 1.695−5.185; OR = 2.965; $p = 1.12 \times 10^{-4}$) and the years of evolution of the DM2 patient (>10 years) with the presence of DPN ($X^2 = 44.755$; 95% CI = 3.948−13.250; OR = 7.233; $p = 2.23 \times 10^{-11}$).

Other clinical characteristics, such as systolic and diastolic pressure, BMI, fasting plasma glucose, cholesterol, triglycerides, and HbA_{1c} were analyzed for all patients. The means of systolic/diastolic blood pressure for the DPN and non-DPN patients were 125.11 ± 12.65/77.13 ± 8.67 mmHg and 121.64 ± 11.27/75.07 ± 6.87 mmHg, respectively. When comparing the means of blood pressure values between both groups of diabetic patients, significant differences were found in both systolic ($p = 0.035$) and diastolic blood pressure ($p = 0.010$).

Analyzing the subjects affected with hyperglycemia (value over ≥126 g/dL), 72 of DPN patients (80%) and 87 non-DPN patients (68%) presented this condition (IC = 1.00−3.56, $p = 0.049$). Hypercholesterolemia (value over ≥200 mg/dL) was present in 66 DPN patients (73%) and 65 non-DPN patients (51%) (IC = 1.49−4.77, $p = 8.15 \times 10^{-4}$). High HbA_{1c} concentrations (value over ≥7%) were present in 75 DPN patients (83%) and 58 non-DPN patients (45%) (IC = 0.861−0.3189, $p = 1.45 \times 10^{-8}$).

Hypertriglyceridemia (value over ≥150 mg/dL) was present in 72 DPN patients (80%) and 100 non-DPN patients (78%). Comparative analysis of subjects affected with hypertriglyceridemia in DPN and non-DPN patients, showed no significant association with the presence of DPN ($p = 0.730$).

Genotypes and DPN Susceptibility

The distribution of VEGF 936 CT gene polymorphism was determined in DM2 patients with DPN ($n = 90$) and without DPN ($n = 128$). The distribution of the homozygous CC, heterozygous CT and homozygous TT genotypes were in concordance with the Hardy-Weinberg equilibrium. The allele and genotype frequencies for each polymorphism were compared in patients with and without DPN. PCR-RFLP analysis showed the homozygous CC as the predominant genotype in 46 of 90 DPN patients, whereas

Table 1. Distribution of different VEFG polymorphism allele and genotypes in type 2 diabetes with or without DPN

	DNP, *n* (%) *n* = 90	Non-DNP, *n* (%) *n* = 128	X^2	*p*
+936 C/T Genotype				
CC	46 (51)	50 (39)	5.506	0.063
CT	32 (36)	66 (52)		
TT	12 (13)	12 (9)		
Allele				
C	124 (69)	166 (65)	0.776	0.378
T	56 (31)	90 (35)		
Dominant				
CC	46 (51)[a]	50 (39)	3.11	0.078
CC+TT	44 (49)[b]	78 (61)		
Over dominant				
CT	32 (36)[c]	66 (52)	5.47	0.019
CC+TT	58 (64)[d]	62 (48)		
Recesive				
TT	12 (13)[e]	12 (9)	0.345	0.358
CT+CC	78 (87)[f]	116 (91)		

[a]OR (CI) = 1.63 (0.94-28.1).
[b]OR (CI) = 0.613 (0.356-1.057).
[c]OR (CI) = 0.52 (0.300-0.90).
[d]OR (CI) = 1.92 (1.109-3-356).
[e]OR (CI) = 1.487 (0.636-3.480).
[f]OR (CI) = 0.672 (0.278-1.573).

in non-DPN patients heterozygous CT genotype was the most frequent genotype, observed in 66 of 128 subjects. No statistically significant association was found between genotype distribution in both populations ($p = 0.063$) (Table 1).

The C allele was the most frequent in both groups (69% in DPN and 65% in non-DPN) (Table 1). No significant difference was found when comparing the allele frequency between both groups of diabetic patients ($p = 0.378$).

To determine the association of the VEGF +936 CT gene polymorphism with DPN, the allele with higher frequency (allele C) was compared with the allele of lower frequency (allele T) by means of three models of inheritance (Table 1).

The distribution of the genotypes according to the dominant (CC vs. CT + TT) and recessive (TT vs. CT + CC) models showed that the homozygous CC genotype and the TT genotype, respectively, are not risk factors for DPN ($p > 0.05$). The heterozygous CT genotype conferred a protective effect according to the over-dominant model (CT vs. CC + TT) (OR = 0.52; 95% CI = 0.300–0.90; $p = 0.019$) (Table 1).

We analyzed the association of the genotypes of DPN subjects with clinical variables according to the different inheritance models (Table 2). There were no statistically significant genotype-clinical associations in any of the models.

Discussion

The prevalence of DPN in the population analyzed in this study was 41%, close to the prevalence of 38% (2.4 millions) reported by the National Health and Nutrition Survey (ENSANUT, 2012) (14). In other studies, conducted in different States of Mexico, higher prevalences have been reported for DPN, for example, 69% in Guanajuato (15). This DPN variation in prevalence is probably due to the type of population analyzed, the variability in the years of evolution of the DM2 and the metabolic control of the patients.

The number of patients with DPN analyzed in this study was in agreement with a power analysis of 80%. The patients had other diabetic complications such as retinopathy and nephropathy, independently of the presence or absence of DPN. However, that information was not considered as part of the descriptive or statistical analyses due to the lack of complete data in the studied population.

Clinical variables between DM2 patients with and without DPN were analyzed (DPN and non-DPN patients). It was observed that age and the years of evolution of the DM2 in patients were risk factors for suffering from DPN ($p < 0.05$). A DM2 patient older than 60 years and with 11 or more years of diabetes evolution has an OR of 2.965 and 7.233, respectively). Furthermore, hyperglycemia, hypercholesterolemia, elevated HbA_{1c} levels, as well as systolic and diastolic hypertension, were risk factors for the development of DPN ($p < 0.05$). These results are in agreement with previous reports that indicate that several

Table 2. Analysis of risk factors for the association with VEGF polymorphism genotypes in type 2 diabetes patients

	VEGF genotypes					
	CT	CC+TT	*p*	CC	CT+TT	*p*
BMI	30.3 ± 5.24	30.66 ± 3.99	0.718	30.31 ± 3.72	30.76 ± 5.11	0.634
HbA_{1c}	9.28 ± 2.14	9.3 ± 2.54	0.967	8.97 ± 2.54	9.64 ± 2.21	0.188
Glucose	183.75 ± 68.97	191.84 ± 70.06	0.599	185.17 ± 67.64	192.93 ± 71.75	0.599
TG	245.94 ± 109.73	222.67 ± 82.00	0.258	217.96 ± 84.59	244.52 ± 99.98	0.176
TC	224.75 ± 48.03	231.17 ± 50.85	0.560	225.78 ± 49.79	232.14 ± 49.95	0.547
SBP	123.13 ± 12.297	126.21 ± 12.81	0.271	124.57 ± 13.11	125.68 ± 12.27	0.675
DBP	78.91 ± 9.95	77.24 ± 8.54	0.387	76.74 ± 8.44	78.98 ± 8.86	0.223

Data shown are mean ± standard deviation. BMI, body mass index (kg/m^2); HbA_{1c}, glycosylated hemoglobin (%); Glucose (mg/dL); TG, triglycerides (mg/dL); TC, total cholesterol (mg/dL); SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg).

factors affect the incidence and severity of DPN, such as longstanding hyperglycemia and the duration of diabetes, while patient age, height, hypertension and dyslipidemia are other contributing factors (2,16,17). The presence of hyperglycemia inhibits endogenous protective factors in vascular tissues, such as VEGF, which plays an important role in vascular homeostasis and delays the progression of diabetic complications (18). The main treatment received by DM2 patients in this study was focused on the control of blood glucose levels and antineuritic drugs to reduce the symptoms of DPN. These treatments did not prevent the patients from presenting DPN.

The duration of diabetes is a risk factor for microangiopathic complications (2,16,17). We showed an association of the duration of diabetes (>10 years) with the presence of DPN (OR = 7.233; $p = 2.23 \times 10^{-11}$). The duration of DM2 was 12.39 ± 5.91 in the DPN group and 8.02 ± 5.87 in the non-DPN group. Unfortunately, the date of diagnosis of DM2 in Mexico usually does not correspond to the real date of onset of DM2, because patients seek medical attention when they already have complications, indicating that they already have endured several years of metabolic unbalance at the time of DM2 diagnosis. A limitation of this study was that patients were not followed up to evaluate the role of VEGF polymorphism in the progression of microvascular complications.

In Mexico there are no genetic studies that determine the relationship of genetic factors with the development of DPN. In our study, the analysis of allele and genotype frequency of the VEGF +936 C/T polymorphism was performed. No significant difference was found in the distribution of genotypes when comparing DM2 population with and without neuropathy (DPN and non-DPN, respectively). This result agrees with reports in Korean patients (19). It is important to mention that in these Korean patients a high frequency of the VEGF +936 TT genotype and high incidence of the T allele were associated with retinopathy. Moreover, high levels of plasma VEGF have been demonstrated in the TT genotype and have been related to the severity of retinopathy. However, plasma levels of VEGF were not associated with diabetic neuropathy (19).

In Chinese populations, the C allele frequency and CC genotype frequency were higher in DPN patients (87.50 and 77.94%, respectively), and the 936C allele of VEGF was reported as a genetic marker susceptible to DPN (19). In our study, the C allele frequency (69%) and CC genotype frequency (51%) in the DPN group were higher than those in the non-DPN group; however, the 936C allele was not a risk factor for the presence of DNP ($p = 0.378$). According to the dominant inheritance model (CC vs. CT + TT) it was observed that the CC genotype is not a risk factor for the development of DPN ($p = 0.078$); however, a tendency to be a risk factor was observed by the proximity of the value of p obtained to the statistically significant value (<0.05).

Allele 936T and CT + TT genotype have been reported as a possible protective genetic marker of DPN (8,20,21). In our study, the 936CT heterozygous genotype was the most frequent in the non-DPN group (52%) and showed a protective effect of DPN ($p = 0.019$) according to the over-dominant model (CT vs. CC + TT).

Conclusion

Our study strongly supports the theory that the +936 C/T (rs3025039) polymorphism of the VEGF gene is related with DPN. The 936CC genotype had a tendency to be a risk factor for the development of DPN, and 936CT genotype showed a protective effect. Moreover, patient age (over 60 years), duration of diabetes (more than 11 years), hyperglycemia, hypercholesterolemia, elevated HbA_{1c} levels, as well as systolic and diastolic hypertension, were risk factors for the development of DPN. Our results warrant a larger scale study. These findings extend our understanding of the spectrum of phenotypic and genotypic expression of VEGF gene markers for future clinical and therapeutic implications in DM2.

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