• Investigation •

Evaluation of VEGF gene polymorphisms and proliferative diabetic retinopathy in Mexican population

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Abstract

• AIM: To assess if the included vascular endothelial growth factor (VEGF) polymorphisms rs3025035, rs3025021 and rs2010963 are associated to proliferative retinopathy in a Mexican population with type 2 diabetes mellitus (T2DM).

• METHODS: A case-control study was conducted in adult individuals with T2DM associated to proliferative retinopathy or non-proliferative retinopathy from Oct. 2014 to Jun. 2015 from the Retina Department of the Asociation to Prevent Blindness in Mexico. The selected patients were adults with a diagnosis of T2DM ≥5y. All subjects had a comprehensive ocular examination and the classification of the retinopathy severity was made considering the Early Treatment Diabetic Retinopathy Study (ETDRS) standardization protocols. Genomic DNA was extracted from whole fresh blood. All samples were genotyped by qPCR for selected VEGF polymorphisms. Hardy-Weinberg equilibrium was calculated by comparing Chi-square values between the expected and the observed values for genotype counts.

• RESULTS: In total 142 individuals were enrolled, 71 individuals with T2DM and associated proliferative retinopathy and 71 individuals with non-proliferative retinopathy. One-sided Fisher's exact test was performed for rs3025021 [OR (95% CI)=0.44(0.08-2.2); *P*=0.25] and rs2010963 [OR (95% CI)=0.63(0.25-1.6); *P*=0.23]. The minor allelic frequencies obtained were 26% for rs3025021, 10% for rs3025035 and 61% for rs2010963. The pairwise linkage disequilibrium between the three SNP was assessed, and was as follows: rs3025021 *vs* rs3025035: D'=1.0, r^2=0.1043, *P*≤0.0001; rs3025021 *vs* rs2010963: D'=0.442, r^2=0.0446, *P*=0.149; rs3025035 *vs* rs2010963: D'=0.505, r^2=0.0214, *P*=0.142.

• CONCLUSION: This is the first analysis involving VEGFA polymorphisms and proliferative diabetic retinopathy in a Mexican population. A major finding of the present study is that none of the polymorphisms studied was significantly associated with proliferative retinopathy. Based on these results, we can infer that different populations have different associations for the same polymorphisms.

• **KEYWORDS:** vascular endothelial growth factor polymorphism; proliferative diabetic retinopathy; non-proliferative diabetic retinopathy; rs3025021; rs3025035; rs2010963

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a multifactorial disorder that has reached epidemic proportions worldwide. The incidence of T2DM is predicted to rise substantially over the next few decades [1-3]. T2DM is characterized by chronic elevation of blood glucose levels due to peripheral insulin resistance[4], pericyte apoptosis is the most specific vascular pathology attributed to hyperglycemia[5-7]. The subsequent pathological damage involve injury to endothelial cell and blood vessels´ basal membrane, which leads to retinal blood supply impairment and ischemia; resulting in subsequent formation of abnormal new vessels (retinal neovascularization)[7]. The ischemic cues, generated on the retina, lead to massive production

of vascular endothelial growth factor (VEGF), a multifunctional cytokine that promotes angiogenesis and a potent mediator of microvascular permeability[8]. VEGF is largely responsible for the pathological production of neo-vessels in diabetic retinopathy; leading to severe complications and irreversible loss of vision[9].

Several studies have shown that susceptibility to proliferative diabetic retinopathy (PDR) also has a genetic component, independent of glycemic control and duration of diabetes [10-12]. Several single nucleotide polymorphisms (SNP) have been described in the VEGF gene, some of which have been reported to increase VEGF expression *in vitro* [13-14]. A series of previous studies reported association of VEGF SNP with PDR and macular edema[15-16].

The SNP, rs3025035, rs3025021 and rs2010963 have previously been associated to PDR in several populations; however to our knowledge there are no studies regarding the behavior of these SNP in a Mexican-based population [17-19]. The aim of this study was to determine whether rs3025035, rs3025021 and rs2010963 SNP in the VEGF gene are associated with PDR in a Mexican population with T2DM.

SUBJECTS AND METHODS

Study Design This case control study was performed at the Association to Prevent Blindness in Mexico Hospital (APEC) and at the School of Medicine of the Autonomous University of Queretaro (SM-UAQ). The internal review board of the Association to Prevent Blindness in Mexico and the Bioethical Committee of SM-UAQ revised and approved this study. This study was conducted in accordance to the tenets of the Declaration of Helsinki and Good Clinical Practices Guidelines. All participants were briefed extensively and signed a written informed consent form before any study procedures were performed. Sensible data was handled in accordance to the health insurance portability and accountability act.

The study enrolled patients with clinical and laboratorial diagnosis of T2DM from Oct. 2014 to Jun. 2015 from the Retina Department of the APEC. All participants were born in Mexico from parents and grandparents whom identified themselves as Mexican mestizos. The inclusion criteria were patients with T2DM, aged 18y or older and diagnosed at least 5y before the time of their enrollment in study. Diagnosis of T2DM was made according to World Health Organization criteria [20]. All of the subjects had a comprehensive ocular examination including a review of the medical history, slit-lamp examination, Goldmann applanation tonometry, dilated fundus examination and seven-field digital fundus photography with fluorescence angiograph. Classification of severity of retinopathy was based according to the Early Treatment Diabetic Retinopathy Study (ETDRS) standardization protocols [13].

DNA Extraction and Genotyping Genomic DNA was extracted from whole fresh blood by using the Wizard Genomic DNA Purification kit (Promega, Madison WI, USA), according to manufacturer instructions. Concentrations of purified DNA samples were spectrophotometrically determined and adjusted to 20-50 ng/mL. Genotyping for rs2010963, rs3025021 and rs3025035 were performed by using TaqMan® SNP Genotyping Assays (catalog numbers C_83116114_10, C_1647365_10 and C_15985281_10, for rs2010963, rs3025021 and rs3025035; respectively, Applied Biosystems, Foster City, CA, USA). PCR reactions were carried out in a total volume of 25 µL containing 20 ng of genomic DNA, 12.5 µL of TaqMan Universal Master mix (concentration of 2×, Applied Biosystems), 1.25 µL of TaqMan SNP Genotyping Assay (concentration of 20×, Applied Biosystems) containing both primers and probes. PCR was performed by using a StepOne Real time PCR system (Applied Biosystems; USA) under the following conditions: one cycle at 60℃ for 30 s, 1 cycle at 95℃ for 10 min followed by 40 cycles at 95℃ for 15 s and 60℃ for 1 min. PCR products were analyzed by StepOne™ Software Ver2.01 (Applied Biosystems, USA) and alleles called with the default algorithm. Random regenotyping were conducted in 10% of the samples to confirm the results.

Statistical Analysis A sample size of 71 per group (142 total) with a case-control ratio of 1:1 was determined by using the OSSE calculator (osse.bii.a-star.edu.sg), which estimates sample size for case-control studies of association of genetic polymorphisms, in order to detect an OR of 2.7 with a significance level of 5%, a 95% confidence interval and a test power of 80%. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software (version 15, SPSS, Inc., Chicago, IL, USA). Continuous and categorical variables are displayed as means ± standard deviation (SD) and percentages, respectively. Differences between categorical variables were analyzed by Fisher´s exact test and the analysis of variance (ANOVA) for continuous variables. $P<0.05$ were considered to be statistically significant. Normal and non-normal distributions were determined by Kolgomorov-Smirnov tests for all variables. Mann-Whitney tests were used to assess the differences between medians. One-tailed Spearman correlation tests were applied to non-normally distributed data. Hardy-Weinberg equilibrium was calculated by comparing chi square values between the expected and the observed values for genotype counts [21]. Linkage disequilibrium was calculated by employing the Cube X online software [22].

RESULTS

A total of 142 patients were enrolled in the study, clinical and demographic data are summarized on Table 1.

There were no statistically significant differences between the NPDR and the PDR groups in terms of age, duration of the diabetes, age of T2DM onset, serum glucose and HbA1c levels. Nevertheless, the means for age, duration of the diabetes, age of T2DM onset were superior in the PDR group.

Table 1 Demographic, clinical and biochemical features studied

Clinical features	PDR (*n*=71)	NPDR (*n*=71)	*P*
Age(a)	67.2±10.8	66±12.3	0.165
Gender (M/F)	46/25	41/30	0.245
Duration of T2DM (a)	14.6±6.6	13.2±7.2	0.213
Age of T2DM onset (a)	52.5±10.9	50±11.7	0.149
Serum glucose (mg/dL)	150.2±68.5	163.7±68	0.210
HbA1c (%)	8.1±2.3	8.2±2.1	0.596

PDR: Proliferative diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; T2DM: Type 2 diabetes mellitus; HbA1c: Glycated hemoglobin type A1c. No statistically significant differences between groups were found.

The risk allele frequencies (RAF) obtained were as follows: 26% for rs3025021, 10% for rs3025035 and 61% for rs2010963. For the three studied SNP, genotypic frequencies were found in Hardy-Weinberg equilibrium (P>0.05), and genotyping call rates were above 99%, with a regenotyping concordance rate >99%. The pairwise linkage disequilibrium between the three SNP was assessed, and was as follows: rs3025021 *vs* rs3025035: D'=1.0, r^2=0.1043, P=7.6×10^{-5}; rs3025021 *vs* rs2010963: D'=0.442, r^2=0.0446, P=0.149; rs3025035 *vs* rs2010963: D'=0.505, r^2=0.0214, P=0.142. Table 2 shows allelic and genotypic frequencies for the three SNP as well as Hardy-Weinberg equilibrium.

As shown in Table 3, association tests with PDR were non-significant for all three SNP. Haplotype analyses were also not significantly associated to PDR (data not shown).

DISCUSSION

This is the first analysis involving VEGFA polymorphisms and PDR in a Mexican population. A major finding of the present study was that none of the SNP studied was significantly associated with PDR.

Previous studies have shown association of these SNP with PDR, although it was not found to be significantly associated with PDR in our study. Al-Kateb *et al*[23] and Abhary *et al*[2] found rs3025021 to be associated to PDR severity in Caucasian population from Canada and Australia, respectively, whereas Yang *et al*[24] found no significant association with PDR in Chinese population. In another study, Awata *et al*[16] found the rs2010963 C allele to be associated with macular edema and the presence of diabetic retinopathy in a Japanese based population study. Nevertheless, his findings contrast with those reported by Freathy *et al*[25] and Yang *et al*[24] who found no association between rs2010963 SNP and PDR in UK, and Chinese populations, respectively. In addition, Churchill *et al*[18] found that the haplotype formed by rs2010963 and rs1570360, was significantly associated with T2DM without retinopathy in a UK sample, thus highlighting the controversial role of the rs2010963 SNP. Regarding this SNP, a Meta-analysis published by Han *et al*[26] confirms the lack of association

Table 2 Hardy-Weinberg Equilibrium (HWE) for rs2010963, rs3025021 and rs3025035 %

SNP	Frequency		HWE	
			Expected frequency	χ^2
rs2010963	*Genotypic*			
	C/C	14.8	15.4	
	C/G	48.9	47.7	0.11
	G/G	36.2	36.8	
	Allelic			
	C	39.2		
	G	60.7		
rs3025021	*Genotypic*			
	C/C	53.3	54.6	
	C/T	41.2	38.5	0.85
	T/T	5.4	6.8	
	Allelic			
	C	73.9		
	T	26		
rs3025035	*Genotypic*			
	C/C	89.5	89.8	
	C/T	10.4	9.9	0.55
	T/T	0	0.2	
	Allelic			
	C	89.5		
	T	10.4		

SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; χ^2: Chi-squared. Allelic and genotypic frequencies for the three SNP included, and depict the genotypic frequencies, which were found in HWE.

Table 3 Association between the presence of rs2010963, rs3025021 and rs3025035 SNP with PDR risk *n* (%)

SNP (Genotype)	PDR	NPDR	*P*	OR (95% CI)
rs3025021				
AA (C/C)	35 (49.3)	62 (55.9)		
Het (C/T)	34 (47.9)	41 (36.9)	0.14	1.46 (0.79 - 2.7)
Hom (T/T)	2 (2.8)	8 (7.2)	0.25	0.44 (0.08 - 2.2)
LRA (C)	52 (73)	53 (74)	0.5	1.053 (0.56 - 1.97)
HRA (T)	19 (27)	18 (26)		
rs3025035				
AA (C/C)	64 (90.1)	99 (89.2)		
Het (C/T)	7 (9.9)	12 (10.8)	0.52	0.9 (0.33 - 2.4)
Hom (T/T)	0 (0)	0 (0)	-	-
LRA (C)	4 (5)	4 (5)	-	1.0 (0.28 - 3.56)
HRA (T)	67 (95)	67 (95)		
rs2010963				
AA (G/G)	22 (31)	43 (38.7)		
Het (G/C)	37 (52.1)	53 (47.7)	0.46	0.87 (0.36 - 2.0)
Hom (C/C)	12 (16.9)	15 (13.6)	0.23	0.63 (0.25 - 1.6)
LRA (G)	31 (43)	23 (33)	0.09	0.65 (0.36 - 1.16)
HRA (C)	40 (57)	48 (67)		

Fisher's exact test was employed for the analysis of this contingency table. The heterozygous and homozygous groups were compared to the ancestral allele group. PDR: Proliferative diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; OR: Odds ratio; AA: Homozygous for the ancestral allele; Het: Heterozygous; Hom: Homozygous for the risk allele. LRA: Low-risk allele; HRA: High-risk allele. No statistically significant associations were evidenced for all three SNP analyzed.

across all studies performed in Asian and Australian populations. Based on these results, we can infer that different populations have different associations for the same SNP.

In our study, there were no statistically significant differences in age, HbA1c and serum glucose levels in the patients from both groups; thus eliminating age, glucose and HbA1c serum levels as a potential bias. These findings could be partially explained considering the limited availability of health care services for the population living in remote and distant locations, which constitutes a major segment of the patients included in this study. Therefore, precarious metabolic conditions would be expected in a significant number of patients regardless of the T2DM age of onset, serum glucose or HbA1c levels.

In addition, the linkage disequilibrium pairwise analyses for rs3025021 and rs3025035 showed that these SNP are simultaneously inherited. The rest of pairwise analyses showed no statistical significance, which strongly suggest that the last SNP (the rs2010963 polymorphism) has an independent hereditary pattern, which has not been previously reported in this population. These findings are expected considering that rs3025021 and rs3025035 are located in contiguous introns (6 and 7, respectively) within the VEGF gene, whereas the rs2010963 is located in the first intron of VEGF gene far upstream from the other SNP location.

Several limitations in this study should be noted, one of the main weaknesses of this study include the low sample size; however, our sample size calculation was performed in order to detect a minimal OR of 2.7, which is reasonable considering previous reports[2,15-16,18]. Another limitation is the lack of clinical data regarding microvascular complications related to secondary diseases (*i.e.* hypertension, dyslipidemia or nephropathy) and pharmacological treatment in the studied population. Nonetheless this lack of information should not alter our results, since the treatment was not our focus and no significant differences in the clinical and biochemical features studied were found between both groups.

In conclusion, previously reported association between these SNP and PDR varies depending on the studied population. The detection of potential associations between polymorphisms and PDR in Mexican population could allow health care providers to offer a more efficient diabetic retinopathy screening process, as well as to offer an early treatment when needed. Larger studies with independent replication are needed with larger sample sizes to confirm and further support these findings and their clinical importance.

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