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Combination of Angiotensin Converting Enzyme Insertion/Deletion (I/D) (rs4646994) and VEGF Polymorphism (+405G/C; rs2010963) Synergistically Associated With the Development, of Albuminuria in Iranian Patients With Type 2 Diabetes

Mohammad Fathi [1]; Abdol Rahim Nikzamir [2,3,*]; Alireza Esteghamati [3]; Manouchehr Nakhjavani [3]; Mir Saeed Yekaninejad [4]

[1]Department of Anesthesiology, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran
[2]Department of Biochemistry, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran
[3]Endocrinology and Metabolism Research Center (EMRC), Vali-e-Asr Hospital, Tehran University of Medical Sciences, Tehran, IR Iran
[4]Department of Epidemiology and Biostatistics, Tehran University of Medical Sciences, Tehran, IR Iran

*Corresponding Author: Abdol Rahim Nikzamir, Department of Biochemistry, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran. Tel: +98-2166948671, Fax: +98-2166948671, E-mail: nikzamirar @ yahoo.com

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Background: Angiotensin-converting enzyme (ACE) insertion/deletion (I/D) and vascular endothelial growth factor (VEGF) polymorphisms have been shown to associate with diabetic nephropathy (DN).
Objectives: We examined the hypothesis that ACE-D and VEGF-G alleles act synergistically in association with DN, in patients with type 2 diabetes mellitus (T2DM).
Patients and Methods: The VEGF (rs2010963) and ACE (rs4646994) genotypes were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 490 T2DM patients. Diabetic patients were classified as T2DM patients with and without albuminuria (control). The PCR and RFLP were used to detect the VEGF and ACE alleles.
Results: A total of 255 consecutive patients with T2DM and microalbuminuria (Group A) and 235 patients with T2DM and normoalbuminuria (Group B) were included in the study. In univariate analysis, the groups were statistically similar for all variables, except for glycated hemoglobin (HbA1c) (P = 0.034), and the frequency of ACE (P = 0.015) and VEGF (P = 0.006) genotypes. Our study showed that the VEGF-G and ACE-D alleles are independently associated with the development of nephropathy. According to our data, the combination of these two risk factors had a significant synergistic effect on the risk of microalbuminuria development.
Conclusions: Our study indicated that ACE-D and VEGF-G alleles can be an independent risk factor for microalbominuria in T2DM patients.

Keywords: Vascular Endothelial Growth Factor; Albuminuria; Diabetes Mellitus; Genetic Polymorphism

1. Background

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD) (1-3). Patients with non-insulin-dependent diabetes mellitus (NIDDM) present a high risk for developing severe complications, such as DN, retinopathy and cardiovascular disease. Studies demonstrating familial clustering of DN, cardiovascular disease and hypertension (1-3) suggest that, in addition to poor glycemic control, genetic factors may affect the susceptibility to the development of diabetic micro- and macroangiopathy (4-7).

The DN arises from the combination of hyperglycemia and hypertension, driving glomerular damage (8). The underlying pathological changes involve thickening of the basement membrane, atrophy, interstitial fibrosis, and arteriosclerosis. This initially results in glomerular hyperfiltration and, subsequently, progressive loss of renal function (9). The DN occurs in 30-40% of patients within 25 years from the diagnosis of diabetes. However, it is not well understood why several individuals with 'poor control' are protected against renal disease (10-12).

Some studies suggest that genetic factors may be involved in the etiology of renal disease in type 2 diabetes mellitus (T2DM). Possible genetic factors are the Angiotensin-converting enzyme (ACE) and vascular endothelial growth factor (VEGF) polymorphisms (13-17). The ACE-D allele has been suggested as a risk factor for coronary artery disease in patients with nephropathy. The ACE-D allele may play a role in the progression of it rather than the susceptibility to DN (18-20). Hyperglycemia is a crucial factor in the development of DN because of its effects on glomerular and mesangial cells. Nevertheless, it is not causative by itself. Hyperglycemia is thought to stimulate VEGF expression and, therefore, act as a mediator of endothelial injury in diabetic patients (21). Studies have shown initially that,

in patients with DN, the degree of neovascularization was increased and correlated with the expression of VEGF and angiopoietin (22).

2. Objectives

We attempted for the first time on Iranian patients with type 2 diabetes, to investigate the association between ACE (rs4646994) insertion/deletion I/D and VEGF (rs2010963) polymorphisms and their possible synergistic effects on the development of albuminuria in patients with T2DM.

3. Patients and Methods

3.1. Patients

This study recruited 490 patients with T2DM (235 patients without albuminuria (Group A) and 255 patients with microalbuminuria (Group B), who consecutively attended the Diabetes Clinic of Imam Khomeini Hospital, affiliated to Tehran University of Medical Sciences (TUMS), Teheran, Iran, between June 2012 and March 2013. Demographic and anthropometric data, including age, sex, duration of diabetes, height, weight in light clothing, and blood pressure (in sitting position after 10 min rest) were collected. Cholesterol, triglyceride, high density lypoprotein-cholesterol (HDL-C), and low density lipoprotein-cholesterol (LDL-C) were determined using enzymatic methods by Parsazmun kit (Parsazmun, Karaj, Iran). The glycated hemoglobin (HbA1c) was assayed by the high-pressure liquid chromatography method. All patients gave their written informed consent and the study protocol was approved by the Ethics Committee of the Tehran University of Medical Sciences, Teheran, Iran.

The diagnosis of diabetes was based on the criteria suggested by the American Diabetes Association. The DN was diagnosed by the presence of albuminuria, defined as an albumin excretion rate $\geq$ 30 mg/24 hour on at least two out of three consecutive occasions during the past 6 months. Urinary albumin concentrations were measured by immunoturbidmetry (Cecile Instruments, Cambridge, United Kingdom). The detection limit was established at 2 mg/L. Hypertension was defined as resting systolic blood pressure (SBP) $\geq$ 130 mmHg and diastolic blood pressure (DBP) $\geq$ 85 mmHg or ongoing antihypertension treatment. After a 12 hour overnight fasting, 10 mL of 15% ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was obtained from each patient. This sample size was calculated for comparing ACE and VEGF alleles' distribution in two groups. To detect 15% difference in allele proportion in two groups, with 90% power and 5% significance level, 220 samples would be needed in each group.

3.2. Genomic DNA Analysis

Five ml of peripheral blood samples were collected in tubes containing EDTA. Genomic DNA was isolated from peripheral blood leukocytes by standard methods and stored at -20°C. Genotyping of the +405G/C; rs2010963 polymorphism in the VEGF gene and I/D (rs4646994) polymorphism in the ACE gene were determined by employing the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and PCR, respectively (23, 24).

3.3. Statistical Analyses

Data are represented as mean and SD in parentheses for numerical variables or frequency and percentage for qualitative variables. The Chi square test was used for assessing the relationship between polymorphisms of the genotype and alleles within two study groups. Also, the Chi square test was applied to determine whether the observed genotype frequencies were consistent with the Hardy-Weinberg equilibrium. Normality of distribution for every quantitative variable which was compared in two groups was assessed with the Kolmogorov-Smirnov test and through visual inspection of the data. Multiple logistic regression analysis models were conducted for evaluating the risk of genotypes on microalbuminuria, adjusting for age, sex, and diabetic duration. Odds ratios and confidence intervals were reported as measures of association for logistic regression models. A $P < 0.05$ was considered as statistically significant. All statistical analyses were performed by an independent biostatistician with SPSS for Windows version 18.0 (SPSS Ins., Chicago, ILL, USA).

4. Results

Table 1 shows the demographic and clinical features of the subjects in the two study groups. The mean age of patients in group B was with about 1.2 years higher compared to the normal group (59.6 vs. 58.4 years, P = 0.10) and also, diabetes duration was with 1.6 years longer in group B, with borderline significance (14.7 *vs.* 13.1, P = 0.075). Almost half of the subjects in group B and 45.1% in the normal group were male. In group B, HbA1c level was significantly higher (8.54% *vs.* 8.21%, P = 0.034). There was no significant difference in other laboratory variables and clinical features within the two groups. The Chi square test revealed that all genotype frequencies are consistent with the Hardy-Weinberg equilibrium (P > 0.20). The distribution of ACE and VEGF genotypes and alleles are depicted by Table 2. For ACE polymorphism, the D allele was significantly more frequent in group B (57.1% *vs.* 48.1%, P = 0.005). The ACE genotype distribution was significantly different between the two groups (P = 0.015). For VEGF polymorphism, the G allele was significantly more frequent in group B (55.1% *vs.* 46.0%, P = 0.004). The distribution of VEGF was significantly different between the two groups (P = 0.006).

Tables 3 and 4 reveal the results of two logistic regression models for evaluating the effect of VEGF and ACE genotypes on the risk of microalbuminuria after adjusting for age, sex, and diabetic duration. In result, the risk of microalbuminuria in those subjects who had GG genotype, was 2.22 times higher compared with the subjects with CC genotype (P = 0.003). Also, those with DD genotype in ACE were 1.96 times were more prone to have

Table 1. Demographic and Clinical Characteristics in Diabetic Microalbuminuric and Normal Subjects [a]

Variables	Normoalbuminuria [b] (n = 235)	Microalbuminuria [c] (n =255)	P Value [d]
Age, y	58.42 (7.62)	59.59 (8.07)	0.101
Gender, male	106 (45.1)	129 (50.6)	0.225
Diabetic duration, y	13.07 (5.69)	14.66 (12.53)	0.075
BMI, kg/m^2	26.40 (4.54)	26.40 (3.87)	0.986
SBP, mmHg	137.71 (22.06)	139.37 (20.70)	0.389
DBP, mmHg	86.62 (10.83)	85.35 (10.02)	0.180
FBS, mg/dL	204.50 (68.62)	204.65 (61.32)	0.980
Cholesterol, mg/dL	209.99 (45.49)	221.64 (101.41)	0.098
Triglyceride, mg/dL	175.90 (70.69)	179.40 (75.79)	0.508
HDL, mg/dL	43.49 (9.39)	42.75 (9.02)	0.385
LDL, mg/dL	118.95 (30.95)	121.59 (32.20)	0.356
VLDL, mg/dL	35.81 (11.33)	37.13 (10.99)	0.194
HbA1c, %	8.21 (1.74)	8.54 (1.71)	0.034
Smoking	52 (22.1)	45 (17.6)	0.214
Hyperlipidemia	84 (35.7)	80 (31.4)	0.306
Hypertension	104 (44.3)	112 (43.9)	0.941
CAD	37 (15.7)	29 (11.4)	0.157

[a] BMI, body-mass index; CAD, coronary artery disease; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA1c, glycated hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure; VLDL, very low density lipoprotein. Data are represented as Mean (SD) or No. (%).
[b] Group A.
[c] Group B.
[d] Non-significant.

Table 2. The Distribution of Angiotensin Converting Enzyme and Vascular Endothelial Growth Factor Genotypes and Alleles in Diabetic Microalbuminuric and Normal Subjects [a]

Variables	Normoalbuminuria [b] (n = 235)	Microalbuminuria [c] (n =255)	P Value
ACE genotypes			
II	61 (26.0)	49 (19.2)	
ID	122 (51.9)	121 (47.5)	0.015
DD	52 (22.1)	85 (33.3)	
ACE alleles			
I	244 (51.9)	219 (42.9)	0.005
D	226 (48.1)	291 (57.1)	
VEGF genotypes			
CC	63 (26.8)	53 (20.8)	0.006
GC	128 (54.5)	123 (48.2)	
GG	44 (18.7)	79 (31.0)	
VEGF alleles			
C	254 (54.0)	229 (44.9)	0.004
G	216 (46.0)	281 (55.1)	

[a] ACE, angiotensin converting enzyme; VEGF, vascular endothelial growth factor. Data are represented as No. (%).
[b] Group A.
[c] Group B.

Table 3. Logistic Regression Model for Evaluating the Effect of Vascular Endothelial Growth Factor Genotypes on Risk of Microalbuminuria Adjusting for Age, Sex and Diabetic Duration [a]

Variables	OR (95% CI)	P Value
Age	1.02 (0.99-1.04)	0.202
Gender, male	1.27 (0.88-1.83)	0.203
Diabetic duration, y	1.02 (0.99-1.04)	0.126
VEGF genotypes		
CC	Referent	Referent
GC	1.23 (0.78-1.93)	0.376
GG	2.22 (1.31-3.74)	0.003

[a] Data are represented as. CI, confidence interval; OR, odds ratio; VEGF, vascular endothelial growth factor.

Table 4. Logistic Regression Model for Evaluating the Effect of Angiotensin Converting Enzyme Genotypes on Risk of Microalbuminuria After Adjusting for Age, Sex and Diabetes Duration [a]

Variables	OR (95% CI)	P Value
Age	1.01 (0.99-1.04)	0.299
Gender, male	1.26 (0.87-1.80)	0.218
Diabetic duration, y	1.02 (0.99-1.04)	0.136
ACE genotypes		
II	Referent	Referent
ID	1.22 (0.77-1.93)	0.394
DD	1.96 (1.17-3.28)	0.011

[a] ACE, angiotensin converting enzyme; CI, confidence interval; OR, odds ratio. Data are represented as. CI, confidence interval; OR, odds ratio.

Table 5. Logistic Regression Model for Evaluating the Effect of Angiotensin Converting Enzyme and Vascular Endothelial Growth Factor on Risk of Microalbuminuria After Adjusting for Age, Sex and Diabetes Duration [a]

Variables	OR (95% CI)	P Value
Age	1.01 (0.99-1.04)	0.287
Gender, male	1.30 (0.90-1.87)	0.165
Diabetic duration, y	1.02 (1.00-1.05)	0.100
ACE/VEGF		
	Referent	Referent
+/-	1.33 (0.61-2.90)	0.473
-/+	1.47 (0.65-3.29)	0.352
+/+	1.68 (1.00-2.82)	0.049

[a] ACE, angiotensin converting enzyme; CI: confidence interval; OR, odds ratio; VEGF, vascular endothlial growth factor., The combination effect of ACE and VEGF ACE ("-": II , "+": ID or DD), VEGF ("-": CC , "+": CG or GG). Data are represented as CI, confidence interval; OR, odds ratio.

microalbuminuria (P = 0.011). Table 5 shows the combination effect of ACE and VEGF, as two risk factors, on microalbuminuria. In this logistic regression model, two polymorphisms are dichotomous variables. According to

this model, the combination of two risk factors had significant synergistic effect on risk of microalbuminuria (OR = 1.68; 95% CI: 1.00-2.82, P = 0.049).

5. Discussion

The role of the VEGF and ACE gene polymorphism in DN is controversial. Both VEGF and ACE play an important role in the pathogenesis of diabetic microvascular complications. In our study, two groups of patients made the object of the research, namely the normoalbuminuria group and the microalbuminuria group, as stated in the previous literature (25-28). In the present study, we investigated the relationship between this risk factor in a population from Tehran, Iran. Our data indicate that the + 405 GG genotype increases the chance of an Iranian individual to develop diabetic albuminuria. This association was also observed after we controlled for other possible risk factors such as: age, sex, and diabetes duration. Therefore, our results suggest that the GG genotype is an independent risk factor for the development of DN in Iranian patients. This was in agreement with results from other trials (29, 30). However, our findings were not in accordance with data obtained in the report of Buraczynska (31) and Ray (32).

We also investigated the relationship between the G allele (GG/CC) and the development of nephropathy in T2DM patients. Our study showed that the G allele was associated with the development of DN. Summers et al. demonstrated a significant increase in the VEGF + 460 CC genotype in patients with progressive glomerular disease (29). In patients with type 1 or T2DM, the likelihood of developing DN is markedly increased in cases where a sibling or parent has DN (33, 34). These studies suggest that VEGF could be a potential mediator of glomerular hyperfiltration and proteinuria in DN (10). Lenz et al. indicated a correlation between urinary VEGF and urinary albumin in type 2 but not in type 1 diabetic patients with renal disease (35). In a study on 426 type 2 diabetic patients from Poland, there was no significant difference in genotype distribution between patients with or without nephropathy (31). The same result was repeated in a study by Aiello et al. in T2DM patients (36).

Also, our study indicated that the ACE polymorphism was significantly associated with the development of albuminuria. In particular, patients with the DD genotype had a nearly two times higher chance of having micro- versus normoalbuminuria compared to those with the II genotype. In the present study, we had the advantage of a close match between the two groups of patients in a long list of potentially confounding variables. It should be noted, however, that there are several reports on type 1 diabetes without statistically significant results (29, 37).

In T2DM, there are even more controversies. The potential effects of the ACE and VEGF gene polymorphism can be viewed from two perspectives: first, development of/susceptibility to albuminuria and second, the severity/progression of albuminuria. To address the former, the best strategy for a cross-sectional study would be to compare patients with micro- to patients with normoalbuminuria. The lack of such a structured approach in a number of available reports is a potential reason for the inconsistency of results. Other reasons include ethnic differences, different criteria used for the classification of albuminuria, and small sample sizes in several studies.

The results of studies which evaluated the development of/susceptibility to proteinuria have been highly varied. There was no significant difference in genotype distribution between patients with and without nephropathy in two studies on 445 and 658 German T2DM patients (29, 38). The same result was repeated in a study on 141 Tunisian T2DM patients (39) as well as in a large study on 3139 French patients (40). Our results are in agreement with these studies. The OR we obtained when comparing these two groups regarding ACE genotypes (DD versus II) was relatively high (OR = 1.96). A number of studies have claimed a significant association of the ACE gene I/D polymorphism with the development of albuminuria in T2DM.

A German study on 331 patients with various stages of DN showed that patients with higher renal risks had a higher frequency of the DD genotype (38). The available literature on the possible effects of the I/D polymorphism on the renal response to ACE inhibitor therapy continues to be controversial (41). Further studies are required to resolve the existing controversies. Although findings in the association between VEGF and ACE gene polymorphism and many disorders are documented, reports on its association to DN are still controversial. Therefore, we attempted to investigate the association between ACE (rs4646994) I/D and VEGF (rs2010963) polymorphisms and their possible synergistic effects on the development of albuminuria in Iranian patients with T2DM for the first time.

We observed that the VEGF GG and ACE DD genotypes alone increased the risk of microalbuminuria in T2DM patients. Also, we clearly demonstrated that the ACE D and VEGF G alleles acted in synergy to increase the risk for development of DN in our patients. Albuminuria was observed in patients after adjustment for conventional risk factors including age, sex, and diabetes duration. The allele and genotype frequencies of VEGF G/C and ACE D/I polymorphisms were different between patients with and without albuminuria. The interaction of ACE with VEGF is of special interest, in view of the important role played by the renin-angiotensin system in renal disorders

Saijonmaa et al. (42) observed that VEGF increases ACE at mRNA and protein levels in cultured human endothelial cells. Saijonmaa et al. (43) showed that atorvastatin completely inhibits VEGF-induced ACE up-regulation in endothelial cells, probably by inhibiting protein kinase C (PKC) activation. This effect was mediated via the mevalonate pathway, and inhibition of both farnesyl-pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP)

were involved. These findings may be an additional beneficial effect of statins in the prevention and treatment of cardiovascular and renal diseases.

Our observations suggested that the interaction of VEGF and ACE may play a role in the pathophysiological processes of the DN and the synergistic interaction between VEGF and ACE variants may inhibit proliferative processes in the microvascular wall. If true, such a synergy could be a future target for the pharmacological intervention and combined treatment with ACE inhibitors and inhibitors of VEGF, if made available, may be of value in the prevention of diabetic complications. We demonstrated that ACE D and VEGF G ok alleles synergistically increase the risk of microalbuminuria in T2DM patients, even after adjustment for other variables. The cross-sectional nature of the present study does not allow us to make conclusions about potential causal relationships, for which longitudinal studies are required.

Because of the sample size in the present study and because Iranians show wide genetic diversities, additional analysis on larger samples are needed to clarify the contribution of ACE D and VEGF G alleles to the development of DN in different Iranian populations and various ethnic groups in the world. The authors are aware of only a few case-control studies, in other populations, that have evaluated whether I/D polymorphism and VEGF G alleles alter the risk of developing albuminuria in diabetic patients or not. However, caution should be exercised in extrapolating an association found in one population to others. The presence or absence of an observed association in any ethnic, racial or geographic population may be related to a number of other factors including gene-gene and gene-environment interactions. We therefore opted for the first time to elucidate the possible association between the synergistically role of ACE D and VEGF G alleles and albuminuria in an Iranian diabetic adult population. Studies of this type can confirm whether the observed associations are consistent over different ethnicities and populations.

One of the limitations of this study was the low number of diabetic patients with DN available for the analysis. Also, the cross-sectional nature of the current study prevents us from drawing cause-and-effect conclusions. Longitudinal studies are needed to determine whether our results came from mere associations or point-to-causal relationships.

Authors' Contributions

Mohammad Fathi: Analysis and interpretation of data, and helped in revision of the manuscript. Abdol Rahim Nikzamir: designed the study wrote the manuscript, and helped to design laboratory methods. Alireza Esteghamati: interpreted the clinical application data, data collection. Manouchehr Nakhjavani: in manuscript revision, and data collection. Mir Saeed Yekaninejad: Statistical analysis.

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