Original Article

Influence of genetic variability at the ACE locus in intron 16 on Diabetic Nephropathy in T1DM patients

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Abstract

Background: Diallelic [insertion/deletion (I/D)] polymorphism in the angiotensin-converting enzyme (ACE) gene has been reported inconsistently as being associated with risk of diabetic nephropathy (DN). **Objective:** To examine the three ACE poly-morphic variants in intron 16 for a possible role in modulating DN in T1DM patients from Kutch region, Gujarat. **Design and setting:** I/D polymorphism in intron 16 of the ACE gene was examined in a case-control group (280 participants with T1DM, case participants n=138; control participants n=142) for association with nephropathy. All recruited individuals were carefully phenotyped and genotyping was performed using polymerase chain reaction and gel electrophoresis methods. Suitable descriptive statistics was used for different variables. **Results:** No departure from Hardy-Weinberg equilibrium was observed in cases or controls. Genetic polymorphism at the ACE locus in intron 16 were significantly associated with nephropathy when analyzed either by genotype or allele frequencies and D/D variant were significantly (p=0.0002) associated with nephropathy at the 5% level. In multivariate analysis, D/D variant had an independent and strongest influence on the micro-albumin excretion (p=0.002, OR=2.11, 95% CI=1.26–4.48). However, it did not independently change the odds of having macroalbuminuria versus microalbuminuria. **Conclusion: Genotype**-associated differences in ACE in intron 16, have functional consequences in genetic susceptibility to diabetic nephropathy in a population with T1DM, and thus represent a potential DN genetic susceptibility locus worthy of replication.

Introduction

Diabetic nephropathy (DN) occurs only in subset of all patients with long-standing type 1 diabetes mellitus (T1DM) and is associated with very high morbidity and mortality, thereby accounts for most of the reduced life expectancy of T1DM patients (1, 2). To optimize patient care, the best approach is early identification of individuals who have higher risk i.e. to identify and target risk factors involved in the development of DN. Besides duration of diabetes, how well it is controlled and other non-glycemic, non-genetic established amendable risk factors, more and more, population based studies; adoption studies and twin studies provide apparent confirmation for genetic contribution. Despite numerous reports

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suggesting a substantial genetic contribution to the susceptibility of DN, causative molecular mechanism(s) are unclear and no major susceptibility genes have been identified so far, but genetic polymorphisms as described with angiotensin converting enzyme gene (ACE; encoding an enzyme that catalyses the conversion of angiotensin I to angiotensin II) are characterized as plausible, logical susceptibility determinant. This has occurred based on the findings that angiotensin II induces progressive renal injury in DN, in part by increasing cellular growth and proliferation and matrix synthesis, leading to glomerular sclerosis (3), as well as by bringing changes in renal hemodynamics, such as an increase in intra-glomerular pressure (3, 4). Furthermore, ACE-inhibitors in clinical and experimental studies prevented structural and functional changes in the diabetic kidney, authenticating its role as an important genetic factor for DN (5, 6).

ACE gene spans 21 Kb (26 exons and 25 introns) on human chromosome 17 at q23 (7), encodes a 1306-amino acid protein, including a signal peptide and manifests as insertion (I) or deletion (D) polymorphism of a 287 bp Alu repetitive sequence, near the 3' end of intron 16 forming three possible genotypes: II, ID or DD (7, 8). This polymorphism was associated with circulating ACE levels (8, 9) and owing to its central role in the regulation of blood pressure, sodium metabolism and renal hemodynamics (3, 4), substantial number of association studies have addressed the role of this polymorphism in the complex etiology of DN, albeit with equivocal results (10-13).

The incongruity in polymorphism association studies might be due to genetic and environmental heterogeneity, as disease prevalence is a consequence of the inter-section of the genetic variation that represents a population with different environmental exposures. Each population is expected to have a different distribution of relative genotype frequencies and a different constellation of possible environmental histories i.e. the presence or absence of an observed association in any ethnic, racial or geographic population may be related to a number of factors including gene-gene and gene-environmental interactions. Therefore, for a

multifactorial disease such as DN, we believe caution should be exercised in extrapolating an association found in one population to others, as the conclusion derived is by no means universal. Hence, we opted to explore the association of three ACE polymorphic variants with DN in T1DM population from Kutch region, Gujarat (India), as it is a region characteristically having high consanguinity rates plus relatively high aggregation rates for familial diseases such as diabetes.

Material and Method

Ethical consideration

The study protocol complied with the Declaration of Helsinki (14) on biomedical research on humans, and was approved by the Institutional Human Research Ethical Committee. Written informed consent was obtained (after providing a detailed study overview) from all participants prior to their recruitment for the study.

Patient selection and study protocol

This was a case-control study conducted from October 2011 to November 2013. Patient selected for this study included unrelated T1DM patients of Kutch origin, being treated and followed up at G. K. General Hospital (Gujarat Adani Institute of Medical Sciences), Bhuj (Gujarat). The history of diabetes mellitus was based on patient self report of a prior physician diagnosis and only patients with past or present proliferative diabetic retinopathy were selected. Proliferative diabetic retinopathy was documented by a description of the history of diabetic retinal disease made by a specialized ophthalmologist during a dilated eye examination using indirect ophthalmoscope. Patients who first underwent laser panphotocoagulation because of severe preproliferative lesions were also included (15); however, patients given focal laser treatment were excluded (15). Other criteria for exclusion were, known proteinuria before the onset of diabetes, patients with drug induced nephrotoxic damage or secondary causes of albuminuria such as obstructive renal disease, renal stone disease and acute urinary tract

infection; patients with, congestive cardiac failure, preexisting macro-vascular condition, any severe illness (such as malignancy, severe infection, respiratory disease, liver disease), impairment of speech, hearing, vision, or cognition, continuous or periodic use of corticosteroids; patients with requirement of insulin after 1 year of diagnosis of diabetes; pregnant females or who had given birth within the preceding six weeks, or any medical condition that prevented participants from adhering to the protocol, lack of approval by physician, geographical distance (distance from the place of residence to the hospital of > 100 km without the possibility of follow-up) and patients showing disinterest or refusal to sign the consent form. In total, 346 patients were recruited in the present study. It was based on consecutive, convenient sampling technique i.e. all the patients who fulfilled the criteria in the period October 2011 to March 2012 were included and it was ensured that 1:1 matching was done.

Patients' examination and measurements

A detailed present and past history of each case was recorded including name, age, sex, address, religion, occupation, economic status, nutritional and personal habits, education, medication and history suggestive of any systemic illness. Each patient was then examined for various anthropometric parameters: Weight (Kg) and height (meters) were measured (using Omron digital body weight scale HN-286 and SECA 206 wall mounted metal tapes respectively). Body Mass Index (BMI) was calculated by Weight (Kg)/ height squared (m^2) (16). Total body fat (%) was measured using bioelectrical impedance analyzer (Omron HBF-362). All anthropometric measures reflect the average of 3 measurements (measured by same person on same instrument to avoid inter-instrument and inter personal variation). Blood pressure (BP) was measured three times (on different days) in the seated position after 10 minutes of rest with a standard manual mercury sphygmomanometer (Diamond Deluxe Industrial Electronics and Products, Pune, India) and stethoscope (3M Littmann, 3M India Ltd, Banglore, India) by auscultatory method (17). The recorded pressure of the three measurements was averaged.

Patients were assigned to a category of hypertensive status according to the criteria formulated by Seventh Report of the Joint National Committee, JNC 7 (18). Age was defined as the age at the time of interview (though no documentary proof had been entertained) and the date of diagnosis of diabetes mellitus was obtained from the patient.

Phenotypic characterization of nephropathy

All recruited participants were screened for renal complication due to diabetes and thus were asked to collect a three 24-hour urine sample, in space of at least 6 months apart (19) for analysis of albumin excretion rate (AER). Urine collection was carried out during unrestricted daily life activity and was tested for AER by immunoturbidimetric assay (CV % : 3.2) (20). The patients were divided into two groups depending on the absence or presence of nephropathy in the form of microalbuminuria (AER of 30 to 299 mg/day) or macroalbuminuria (AER $\geq$ 300 mg/day) (19, 21). Patients were considered cases if the AER was $\geq$ 30 mg/day in at least two of the three 24-h collections (22); all others were considered as controls.

Sampling and biochemical analysis

After an overnight fast of 12 hours, venous sampling was done for biochemical determinations and for isolation of DNA. Serum and plasma was separated by centrifugation of blood sample and were subjected for analytical procedures. Glucose (Glucose oxidase-peroxidase method, CV % : 3.4) (23), cholesterol (Cholesterol oxidase-peroxidase method, CV % : 3.9) (24), triglycerides (Enzymatic method, CV % : 3.6) (25), HDL-C (Phosphpotungstic method, CV % : 4.7) (26), LDL-C (CV % : 3.6) (27), HbA1c (Immunoturbidimetric method, CV % : 3.9) (28) and creatinine (modified Jaffe's method, CV % : 2.5) (29) were measured in fully automated analyzer (Bayer express plus).

DNA Extraction

Genomic DNA was extracted from peripheral blood leukocytes using commercially available DNA extraction and purification kit from GeNei Diagnostics (Banglore, India) based on standard proteinase K

technique (30). It involves 3 steps namely lysis, washing and elution:

I. Lysis

1. Add 200 µl of blood in a 1.5 ml vial and add 50 µl of proteinase K solution (20 mg/ml) in it.

2. Add 200 µl of lysis buffer to the sample and vortex vigorously for 20 seconds. Incubate it at 70 degree centigrade for 15 minutes.

3. Add 4 µl of RNAase A (100 mg/ml), vortex briefly and incubate it at room temperature for 5 minutes.

4. Add 210 µl of absolute ethanol and vortex briefly.

5. Take one DNA column and place in a 2 ml collection tube. Load the ethanol – sample mixture.

6. Centrifuge at 11000 rpm for 1 minute.

II. Wash

1. Place the DNA column in a fresh 2 ml collection tube and add 500 µl of diluted wash buffer I (1 ml of concentrated wash buffer I with 3 volumes of absolute ethanol).

2. Centrifuge at 11000 rpm for 1 minute. Discard the collection tube with flow through.

3. Place the DNA column in a fresh 2 ml collection tube and add 500 µl of wash buffer II (1 ml of concentrated wash buffer I with 3 volumes of absolute ethanol).

4. Centrifuge at 11000 rpm for 1 minute. Discard the collection tube with flow through.

5. Open the DNA column and place it in a fresh 1.5 ml vial, incubate at 70 degree centigrade for 2 minutes to ensure the complete removal of ethanol.

III. Elution

1. Place the DNA column in a fresh 1.5 ml sterile vial. Add 100 µl of pre warmed (70 degree centigrade) elution buffer to the centre of DNA column.

2. Incubate at room temperature for 1 minute. Centrifuge at 11000 rpm for 1 minute.

3. DNA will be eluted in sterile vial.

Eluted DNA was either stored at –20°C or amplified immediately. All DNA samples were amplified within three days following extraction. Before amplification, quantity of DNA in each sample was assessed by measuring the absorption at 260 nm (using molar extinction coefficient of double stranded DNA: 0.020 µg/ml/cm) (31) in a standard spectrophotometer (UV-VIS double beam – 2205, Systronics, Ahmedabad, India).

Determination of genotypes of ACE gene I/D polymorphism

Two oligonucleotide primers, forward: 5'-CTG GAG ACC ACT CCC ATC CTT TCT T-3' and reverse: 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3' based on the flanking sequences of the I/D region on the intron 16 of ACE gene were used to amplify the corresponding DNA fragments by polymerase chain reaction (PCR) (32). Amplification was carried out in a DNA Thermal Cycler (2720, Applied Biosystems) in a final reaction volume of 50 µl containing 50 ng genomic DNA, 20 pM each primer, 2.5 mM each deoxyribonucleotides triphosphate, 1U thermus aquaticus DNA polymerase, 1.5 mM magnesium chloride, amplification buffer contained 20 mM Tris-hydrocholric acid and 50 mM KCl. The thermocycling profile consisted of one minutes of initial denaturation at 94°C followed by 30 cycles of amplification of denaturation at 95°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. Using agarose gel (1%) electrophoresis, amplified products were separated and distinguished using ethidium bromide under UV light as a 190 bp fragment in the absence of an Alu repeat insertion and a 490 bp fragment in the presence of the insertion (genotypes described as II-490 bp, ID-490+190 bp, and DD-190 bp).

Because the D allele in heterozygous samples is preferentially amplified, mistyping of ID heterozygote as D homozygotes may occur – as the extent of amplification is related to the size difference between the allelic PCR products (i.e. shorter ones are preferentially amplified), because longer PCR products (I allele) are less efficiently denatured than shorter products (D allele) (33). Thus, the probability of a complete primer extension is greater for the shorter products (DD) (33). As a result, preferential amplification of the shorter allelic PCR product from a heterozygote may occur. To avoid any mistyping of ID as DD, each sample which had the DD genotype was submitted to a second, independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (forward 5'-TCG GAC CAC AGC GCC CGC CAC TAC-3' and reverse 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3') (34) with identical PCR conditions except for an annealing temperature of 67°C. The feedback produced a 335-bp amplicon only in the existence of an I allele and no product in samples homozygous for DD (34). All listed laboratory analysis was performed at central research laboratory of Gujarat Adani institute of medical sciences, Bhuj (Gujarat) and quality was controlled using standard solutions.

Statistical analysis

The statistical analyses were performed using a Statistical Package for Social Sciences for Windows version 15.0 (SPSS Inc, Chicago, Illinois, USA). Data were expressed as mean±SD (continuous variables), or as percentages of total (categorical variables). Prior to hypothesis testing, data were examined for normality. Non-normally distributed variables were logarithmically transformed before analysis. Two-group comparisons were made using chi-square (χ^2) for categorical variables and Student's t tests or one-way ANOVA for continuous variables.

The distribution of alleles in studied groups was tested for fitting to the Hardy–Weinberg equilibrium (HWE) (using web base program: http://www.oege.org/software/hwe-mr-calc.shtml) (35) through testing the difference between observed and expected frequencies of genetic variants using the χ^2 goodness-of-fit test. In addition, the strength of the association between DN and the ACE I/D polymorphism was esti-mated using ORs (with the corresponding 95% CIs). The ORs were also performed for a dominant model [(DD% + ID%)Vs II%], a co-dominant model [ID% Vs (II% + DD%)] and a recessive model [DD% Vs (II% + ID%)]. Multivariate logistic regression analysis was employed to determine the relations of gene polymorphisms and DN. Associations were expressed as adjusted OR with 95% CI. For all analyses, two-sided probability values < 0.05 were considered statistically significant.

Results

The mean acceptance rate of this protocol was 81.03% (n: 346) by eligible patients (n: 427) and during the study period 66 patients were lost to follow-up. The subgroup and clinical characteristics of the remaining 280 study participants are presented in Table I. The mean reported duration of diabetes mellitus for normo-, micro-, and macro-albuminuria were 29.4±5.1, 24.6±4.9 and 25.8±3.7 years respectively and there were no significant differences in age (p≥0.061), total body fat percentage (p≥0.253), BMI (p≥0.051), lipid parameters (p≥0.053) and sex distribution between groups (p≥0.577). Nephropathy patients had a significantly higher levels of HbA1c (p≤0.034), had diabetes for shorter duration (p≤0.0001), together with higher plasma creatinine (p≤0.038) compared to controls, and more severe albuminuria was significantly associated with higher levels of plasma creatinine (p<0.015) as well as glycated hemoglobin (p<0.042). Systolic and diastolic BP, the number of antihypertensive treatments, and the proportion of patients on ACE inhibitors increased with the stage of renal involvement. Only 15.21% (n: 21) of the cases were not receiving anti-hypertensive medications. Two or more antihypertensive drugs were being taken by 61.59% (n: 85) of the nephropathy patients.

The distribution of ACE genotypes was significantly different between the groups {microalbuminuria vs controls: (x^2: 10.23, 2 d.f. p=0.006); macroalbuminuria vs control: (x^2: 9.53, 2 d. f. p=0.0085)}. The difference was due to significantly higher frequency of ACE

TABLE I : Characteristics of participants.

| Parameters | Control (n:142) Normoalbuminuria Mean±SD | Cases (n:138) | |
		Microalbuminuria (n : 77) Mean±SD	Macroalbuminuria (n : 61) Mean±SD
Age (years)	48.60±9.6	46.71±10.2	49.86±9.4
Sex, males; n (%)	83 (58.45%)	48 (62.33%)	37 (60.65%)
Active smokers, n (%)	34 (23.94%)	21 (27.27%)	15 (24.59%)
Duration of DM (years)	29.4±5.1	24.6±4.9‡	25.8±3.7§
Creatinine (mg/dl)	0.86±0.25	1.04±0.46†	1.21±0.58§††
BMI (kg/m^2)	24.1±3.3	23.2±3.5	23.8±2.7
SBP (mmHg)	134.87±14.22	138.86±15.14*	143.91±13.12§††
DBP (mmHg)	84.03±5.69	85.42±4.14*	87.11±3.56§††
HbA1C (%)	8.2±1.1	8.6±1.6‡	9.1±1.8§††
TG (mg/dl)	162.45±15.11	161.12±18.60	164.66±19.34
Total Cholesterol (mg/dl)	207.93±20.48	210.48±17.45	213.64±20.62
HDL-C (mg/dl)	43.11±2.31	42.17±3.26	42.26±3.61
LDL-C (mg/dl)	119.56±15.71	123.87±17.57	125.87±15.34
Total body fat %	23.49±4.01	23.22±5.24	24.05±3.08

*p<0.05 (Microalbuminuria Vs Normoalbuminuria); †p<0.01 (Microalbuminuria Vs Normoalbuminuria); ‡p<0.0001 (Microalbuminuria Vs Normoalbuminuria); §p<0.0001 (Macroalbuminuria Vs Normoalbuminuria); ††p<0.05 (Macroalbuminuria Vs Microalbuminuria).

D/D homozygotes {(Microalbuminuria: 38.96% vs. 19.71%, OR=2.598, p=0.0002) (Macroalbuminuria: 39.34% vs. 19.71%, OR=2.641, p=0.004)}, and lower frequency of heterozygote ACE I/D {(Microalbuminuria: 38.96% vs. 57.04%, OR=0.481, p:0.011) (Macroalbuminuria: 37.70% vs. 57.04%, OR=0.456, p=0.012)}. Regarding allele frequencies, cases with DN had significantly higher frequencies of the mutant D allele {OR (CI) =1.502 (1.076–2.098), p<0.017} (Table III). Albeit the genotype distribution resulted in a higher frequency of the D allele in the cases than controls, testing genetic equilibrium between the observed and expected genotypes using Hardy – Weinberg equilibrium showed ACE genetic variants were confirming to the law (Table II). In the control group, the genotype frequencies did not differ significantly from values of 38.04 II, 70.91 ID and 33.04 DD predicted from HE equation [The x^2 for departure from equilibrium was 2.874 (1 d.f., p=0.090)]. In group of cases, the x^2 for departure

from equilibrium was 3.02 (1 d.f., p=0.0824) and 3.09 (1 d.f., p=0.0788) in microalbuminuric and macroalbuminuric patients respectively with predicted genotype frequencies of 13.3 II, 37.4 ID, 26.3 DD and 10.66 II, 29.68 ID, 20.66 respectively.

Alliance of ACE I/D polymorphic variant to the risk of nephropathy, was discovered first at the univariate {for D/D variant (OR=2.598, p=0.0002, Table IV)} and then at the multivariate level. Multivariate analysis con-firmed the association of D/D variant with microalbu-minuria (p=0.002, OR=2.11, 95% CI=1.26–4.48, Table V) presence, after adjustment for the covariates, implying a risk of approximately 2.1 times higher than for those homozygous for the I allele. However, it did not independently change the odds of having macroalbuminuria versus microalbuminuria (Table V). The other variable(s) with significant, independent association with microalbuminuria were HbA1c (p=0.048) and creatinine (p=0.026) (Table V).

TABLE II :   Genotype distribution of ACE I/D polymorphism in T1DM patients
with nephropathy and T1DM patients without nephropathy.

| | Genotype frequencies | | | | |
	II	ID	DD	x^2	p
Normoalbuminuria (n: 142)	33(23.23%)	81(57.04%)	28(19.71%)	2.87	0.090
Microalbuminuria (n: 77)	17(22.07%)	30(38.96%)	30(38.96%)	3.02	0.082
Macroalbuminuria (n: 61)	14(22.95%)	23(37.70%)	24(39.34%)	3.09	0.079

TABLE III : Comparison of allele frequencies of ACE I/D polymorphism in T1DM patients with nephropathy (cases) and T1DM patients without nephropathy (controls).

		Cases vs Controls			
Allele	Controls {n : 284 (%)}	Case {n : 276 (%)}	95% CI	p	OR
ACE D	137 (48.23)	161 (58.33)	1.076–2.098	0.017	1.502
ACE I	147 (51.76)	115 (41.66)	0.477–0.929	0.017	0.666
		Microalbuminuria vs Normoalbuminuria			
Allele	Normoalbuminuria {n : 284 (%)}	Microalbuminuria {n : 154 (%)}	95% CI	p	OR
ACE D	137 (48.23)	90 (58.44)	1.015–2.242	0.042	1.509
ACE I	147 (51.76)	64 (41.55)	0.446–0.989	0.042	0.663
		Macroalbuminuria vs Normoalbuminuria			
Allele	Normoalbuminuria {n : 284 (%)}	Macroalbuminuria {n : 122 (%)}	95% CI	p	OR
ACE D	137 (48.23)	71 (58.19)	0.973–2.293	0.066	1.494
ACE I	147 (51.76)	51 (41.80)	0.436–1.028	0.066	0.669

TABLE IV : Matched odds ratio.

Genotypes	Microalbuminuria (n :7 7)				95% CI		Macroalbuminuria (n : 61)				95% CI	
	n	t	p	OR	Lower Bound	Upper Bound	n	t	p	OR	Upper Bound	Lower Bound
II	17 (22.07%)	0.195	0.845	0.935	0.481	1.819	14 (22.95%)	0.043	0.965	0.983	0.482	2.006
ID	30 (38.96%)	2.555	0.011	0.481	0.273	0.846	23 (37.70%)	2.527	0.012	0.456	0.246	0.843
DD	30 (38.96%)	3.777	0.0002	2.598	1.402	4.816	24 (39.34%)	2.938	0.004	2.641	1.365	5.106

TABLE V : Comparison between the groups by multivariate regression analysis.

Covariate	Microalbuminuria Vs Normoalbuminuria			Macroalbuminuria Vs Microalbuminuria		
	p	OR	95% CI	p	OR	95% CI
Ahir community						
Age	0.45	0.87	0.36–1.6	0.434	0.88	0.41–1.63
Sex	0.681	0.59	0.66–1.39	0.886	0.78	0.33–1.47
SBP	0.077	1.16	0.92–2.38	0.075	1.21	0.77–2.26
DBP	0.074	1.21	0.76–2.23	0.063	1.24	0.77–2.27
Creatinine	0.026	1.73	0.96–2.92	0.078	1.37	0.71–2.39
HbA1c	0.048	1.56	0.93–2.75	0.084	1.21	0.63–2.19
DD Vs other genotype	0.002	2.11	1.26–4.48	0.441	0.92	0.36–2.12
ID Vs other genotype	0.059	0.88	0.36–1.54	0.643	0.85	0.49–1.68

Analysis of the association under various genetic models revealed that ACE I/D polymorphic variant contribute to DN susceptibility under recessive mode only [DD% Vs (II% + ID%)] {(For microalbuminuria: x^2: 9.23, OR (95% CI) = 2.60 (1.40–4.82), p=0.002) (For macroalbuminuria: x^2: 8.243 OR (95% CI) = 2.64 (1.37–5.11), p=0.004)} (Table VI). {when comparison of homozygous carriers of D variant plus ID heterozygote (DD + ID) versus homozygous carriers of I variant (II) (dominant model) were made, the OR was 1.07 (p=0.845) and 1.02 (p=0.964) for micro and macro-albumin excretion respectively. Whereas

TABLE VI : Analysis of genetic risk factors under dominant, co-dominant and recessive mode.

	Dominant model [(DD% + ID%)Vs II%]	Co-dominant model [ID% Vs (II% + DD%)]	Recessive model [DD% Vs (II% + ID%)]
Microalbuminuria Vs Normoalbuminuria			
p	0.845	0.059	0.002
OR	1.07	1.52	2.60
95% CI	0.55–2.08	1.02–2.29	1.40–4.82
Macroalbuminuria Vs Normoalbuminuria			
p	0.964	0.061	0.004
OR	1.02	1.51	2.64
95% CI	0.50–2.07	0.98–2.35	1.37–5.11
Macroalbuminuria Vs Microalbuminuria			
p	0.903	0.97	0.963
OR	0.95	0.99	1.02
95% CI	0.43–2.13	0.64–1.54	0.51–2.02

when ID heterozygote (ID) versus homozygous carriers of D variant plus homozygous carriers of I variant (DD+II) (Co-dominant model) were made, the OR was 1.52 (p=0.059) and 1.51 (p=0.061) for micro and macroalbuminuria condition respectively (Table VI).

Discussion

Given the preceding ambiguous studies on the relationship between the ACEI/D polymorphism and DN, we examined its role in the development of micro-albumin excretion and severe nephropathy using a case control approach and found that DD genotype, to be significantly higher in cases than in control participants (p<0.05), is an independent risk factor for development of renal disease in T1DM patients. This result is consistent with previous studies (12, 13, 36-38) and extends them by documenting the deleterious effect of D/D variant in Kutchi T1DM patients. However, it is at variance with one of the first published studies (39), recent meta-analysis (10), and with other studies (40, 41). This discrepant finding among studies may be attributed for a number of reasons: different races, methods of quantitation, study design, statistical power and the heterogeneous phenotypic characterization of cases and controls.

The strength of this study lies in the selection criteria of patient cohort to study the possible role of variable

of interest with the condition in question - as the issue of whether proteinuria is due to diabetes or some other etiology remains debatable, but it appears unlikely that the proteinuria was the result of T1DM-unrelated causes in our group of patients as we excluded the patients who had proteinuria/renal disorders before their diabetes was diagnosed, thus we confined our study to diabetic kidney diseases. Furthermore, we included only those patients that had proliferative retinopathy status, thus minimizes the possibility of misclassification of diabetic individuals as having no nephropathy because of diabetes duration strengthening the notion that the nephropathy was the result of diabetes, more so than unrelated causes. Also, no statistically significant deviation was found from the expected genotype distribution according to the HW equilibrium in any of the groups (Table II), which suggest that for a (I/D) polymorphism, genotype frequencies will remain stable throughout life, thus rules out the possibility of survival bias. Since, any decrease observed for a particular genotype may suggest that alleles for that genotype carry an increased risk of mortality past the age of reproduction (42).

Univariate analysis in the current study revealed a positive relationship between the severity of clinical renal involvement and BP, creatinine as well as HbA1c (Table I), which is in conformity with common clinical observations in diabetic kidney disease (43-45). Duration of the disease was significantly different between the two groups (i.e. cases vs controls), with

control diabetes group having a longer duration, which ensured not only the choice of appropriate controls but also provided the impetus for ACE I/D polymorphic variant (genetic factor) to modulate the risk of renal complications in diabetes, which was discovered first at the univariate level that ACE D/D homozygotes was significantly associated with microalbuminuria (OR=2.598, p=0.0002, Table IV) and macroalbuminuria (OR=2.641, p=0.004, Table IV) presence, implying a risk of approximately 2.5 higher than the other genotypes.

In order to assess the independent role of ACE (I/D) gene polymorphism on the susceptibility and severity of DN, we employed multivariate regression, in which we included age, and sex (as clinically significant variables) along with variables with positive significant associations in univariate analysis (B.P., creatinine and HbA1c) in the covariate list of regression analysis. Results showed that the DD genotype was a significant and the strongest independent predictor of microalbuminuria (Table V). However, when analysis was done to address the second prospective i.e. to assess the potential effects of ACE gene polymorphism on severity of DN (by comparing macroalbuminuria group with microalbuminuria patients), it did not independently change the odds of having macroalbuminuria versus microalbuminuria (Table V), thereby demonstrating that ACE genotype are of value in predicting DN development rather than DN severity and was substantiated by comparable allele or genotypic distribution between the two DN groups. In particular, we identified that, patients with the DD genotype has nearly 2.1 times higher chance of having microalbuminuria than those with the II genotype (Table V), while the ID genotype did not alter the risk significantly, a pattern suggestive of recessive mode of inheritance in allele D of the ACE gene. Same was validated by analysis of genetic risk factors under dominant, co-dominant and recessive model (Table VI).

Thus, the findings from this study indicate the contribution of genetic variability at the ACE locus to the heritable part of risk for DN, and is supported by studies examining DN in families with multiple siblings with T1DM (46-48) along with reports from middle east Asia (12), India (49, 50) and Mexico

(13), unlike those of Dutch (40) or Korean population (51), suggesting an ethnic difference. It is also possible that ACE I/D polymorphism affects the outcome through a linkage disequilibrium with other causative genes or polymorphisms, as a result, positive association will be found in populations with tighter linkage but not in populations with weaker linkage (52). Therefore, the difference between the significant association among one population and null effects among other may not be surprising. To reconcile the existing discrepancies, further large - scale prospective cohort studies are needed in which diabetic patients with and without nephropathy are genotyped for other putative confounders at the ACE locus besides I/D, which may eventually provide a better, comprehensive understanding of the association between the ACE I/D polymorphism and nephropathy risk.

In the interim, since I/D polymorphism is in the intronic region of the ACE gene (8), these findings indicate that the ACE gene I/D polymorphism is a valuable prognostic factor of the risk for the onset of renal complications in T1DM patients. This is consistent with observations made in other types of renal disease: the slope of GFR decline is steeper in patients with the DD versus the II genotypes in patients affected by IgA nephropathy (53, 54), and graft survival after kidney transplantation is better in genotype II patients (55). Taken together, within the limitations of available data, the results of our study are particularly relevant and credible, as we had sufficient statistical power, patients had a poor metabolic control, were on different medications (ACE-I/or other antihypertensive, hypoglycemic and lipid lowering), which reflects the general situation for patients with DN (56). Consequently, our results are directly applicable in a current clinical setting.

Nonetheless, this study has few limitations. Firstly, the design was cross-sectional and therefore cannot provide causal relationship, for which longitudinal studies are required. Secondly, the difference in the distribution of genotypes between cases and controls could have resulted from unintentional, unrecognized population stratification. Despite this population being recruited from a relatively monoethnic society, when

compared to societies with high degrees of ethnic admixture, it is important to recognize the constraints of case-control studies. Thirdly, the proxy definition of diabetes mellitus was used in the study and auto antibodies screening such as Anti-GAD (glutamic acid decarboxylase) analyses were not assessed for patients. Other limitations include those inherent to patient reliability or compliance in complete urine collection.

Conclusion

Our data, obtained using case-control approach, confirm that the risk of DN (but not extent of DN severity) in T1DM is influenced by genetic variability at the ACE locus. Thus, this polymorphism can be used as a valuable indicator for the susceptibility to DN among affected families, who can be counseled to avoid other interactive environmental risk factors.

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