Genetic Predisposition to Diabetic Nephropathy: Evidence for a Role of ACE (I/D) Gene Polymorphism in Type 2 Diabetic Population from Kutch Region

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Abstract Genetic polymorphism as described with angiotensin-converting enzyme gene has been proposed as a putative mediator of diabetic nephropathy. We substantiate the hypothesis that genetic variants of the ACE have significant impacts on diabetic nephropathy. To assess the possible association between the three ACE polymorphic variants and DN in an ethnically homogeneous type 2 diabetic population from Kutch region. A 287-bp insertion/deletion polymorphism in intron 16 of the ACE gene was examined by polymerase chain reaction using a case–control approach conducted with 309 unrelated type 2 diabetic patients of Kutch origin (159 Ahir and 150 Rabari, with ≥ 10 years duration of T2DM). Of the patients, 143 had nephropathy {AER ≥ 30 mg/day (Ahir, $n:73$ and Rabari, $n:70$)} and were considered as cases; all others { $n:166$ (86 Ahir and 80 Rabari)} were normoalbuminuric (AER < 30 mg/day) and were treated as controls. Suitable descriptive statistics was used for different variables. Genotype frequencies in all groups were all in accordance with the Hardy–Weinberg equilibrium. Genotypic distribution was significantly different between cases and controls (Ahir: $\chi^2:8.87$, 2 d.f. $p = 0.0118$; Rabari: $\chi^2:11.01$, 2 d.f. $p = 0.0041$). Multivariate logistic regression analysis revealed that DD genotype was a significant and strongest independent predictor of microalbuminuria (Ahir: $p = 0.0362$, OR = 2.65, 95 % CI 1.89–6.36; Rabari: $p = 0.024$, OR = 2.81, 95 % CI 1.9–6.65). However, it did not independently change the odds of having macroalbuminuria versus microalbuminuria. Analysis of the association

under various genetic models revealed that ACE I/D polymorphic variant contribute to DN susceptibility under recessive mode only. Genetic variation at the ACE locus as D/D variant in intron 16, contribute to an increased risk of nephropathy in T2DM patients but not extent of DN severity, and thus this polymorphism might be considered as genetic risk factors for DN among patients with type 2 diabetes.

Keywords ACE I/D · Polymerase chain reaction · Type 2 diabetes mellitus

Introduction

Diabetic nephropathy (DN), is a multifactorial and polygenic disorder that occurs only in a third to a quarter of all patients with long-standing type 2 diabetes mellitus (T2DM) [1], which is the largest single cause of end-stage renal disease in recent years [2], and a medical catastrophe of worldwide dimensions. This is a warning sign for health care system to be vigilant not only for adequate management, but it is of importance to identify the individuals who have higher risk i.e. to identify and target risk factors involved in the development of DN. Besides conventional modifiable (hypertension, smoking, hyperlipidemia, hemodynamic changes) risk factors, non-modifiable (race, genetic background) factors are also widely considered to contribute to an inherent susceptibility to this disease. Among data supporting a strong heritable component is the clustering of diabetic nephropathy in families [3–5] and the large variation in its prevalence among different population [6–9].

The genetic basis of renal complication in diabetes is heterogeneous, suggesting that the contribution from individual genetic factors is modest. During the past decades, despite huge amount of studies suggesting for several

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candidate genes, no major susceptibility genes have been identified so far, but numerous and extensive analysis in animal models [10] and human studies [11–13] have evidenced that renin–angiotensin–aldosterone system (RAAS) has a crucial role in diabetic kidney disease, with special attention has been focused on angiotensin-converting enzyme (ACE) gene, encoding a key enzyme in the RAAS that catalyses the conversion of angiotensin I to angiotensin II [14]. 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 [15], as well as by bringing changes in renal hemodynamics, such as an increase in intra-glomerular pressure [16, 17]. Blockade of RAAS by ACE-Inhibitors and angiotensin receptor blockers (ARBs) in clinical and experimental studies prevented structural and functional changes in the diabetic kidney, further establishing its role in DN [18–20].

ACE gene spans 21 Kb (26 exon) on human chromosome 17q23 [21, 22], encodes a 1306-amino acid protein, including a signal peptide and manifests a 287-bp repeated Alu sequence insertion (I) or deletion (D) polymorphism in intron 16 forming three possible genotypes: II, ID or DD [22, 23]. This polymorphism was associated with circulating ACE levels [23–25] and owing to its central role in the regulation of blood pressure, sodium metabolism and renal hemodynamics [16, 17], it is reasonable to hypothesize that genetic variation of ACE I/D contributes to the development of DN and numerous studies have addressed the role of this polymorphism in the complex etiology of DN, albeit with conflicting results i.e. several studies have demonstrated [26–31] or refuted [32–34] the role of ACE D variant as candidates for the development of DN. The discrepant finding among studies is attributed to genetic and environmental heterogeneity among different populations. Therefore, 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 factors including gene–gene and gene–environmental interactions. Indeed, diversity of genetic structure and way of life may lead to different conclusions even in the same country, particularly for multifactorial diseases such as DN and since it has been well established that the Indian population is a mosaic of subpopulations, each region, having its own genetic structure with significant genetic distances between neighboring populations. We therefore opted for the first time to elucidate the possible association between the three ACE polymorphic variants and DN in an ethnically homogeneous Kutchi diabetic adult population, as there is a paucity of such studies in Indian population more so specifically in this region.

Primary outcome for the result was to assess the presence of genetic polymorphisms (ACE I/D) in patients with DN. For that purpose a case–control study was performed of patients with DN and normoalbuminuric controls. Secondary outcomes were to determine the effect of gene polymorphisms on susceptibility and severity of DN. For that purpose both univariate and multivariate analyses were performed.

Materials and Methods

Ethical Consideration

The study protocol complied with the Declaration of Helsinki [35] 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.

Selection and Description of Participants

This was a case–control study conducted from September 2011 to April 2013. Patient selected for this study included unrelated adult T2DM patients of Kutch origin belonging to Ahir and Rabari community, being treated and followed up in the diabetic clinics at G. K. General Hospital (Gujarat Adani Institute of Medical Sciences), Bhuj (Gujarat). For the present study only patients with at least 10 years duration of T2DM were selected, to avoid misclassification of diabetic individuals as having no nephropathy because of diabetes duration [36]. The history of diabetes mellitus was based on patient self report of a prior physician diagnosis and was under treatment with oral anti-diabetic agents and/or insulin. In order to exclude patients at low risk of diabetic nephropathy, those without evidence of renal disease despite more than 20 years of diabetes [36] were not eligible to participate in the study. 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, suffering from a serious diabetes complication, continuous or periodic use of corticosteroids; patients with type 1 presentation, defined as diabetic ketoacidosis, acute presentation with heavy ketonuria ($>3+$), or continuous requirement of insulin within 1 year of diagnosis [37]; pregnant females or who had given birth within the

preceding 6 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, 391 patients (Ahir, *n*: 203 and Rabari, *n*: 188) 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 September 2011–December 2011 were included and it was ensured that 1:1 matching was done.

Participants' Examination and Measurements

All patients were studied as outpatient. Patients were interviewed for medical and nutritional history. Present and past history of each case was recorded in detail regarding their general information i.e. 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 (m) 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) [38]. Waist circumference was assessed in the standing position, midway between the highest point of the iliac crest and the lowest point of the costal margin in the midaxillary line. Hip circumference was measured at the level of the femoral greater trochanter. Total body fat (%) was measured using bioelectrical impedance analyzer (Omron HBF-362). All anthropometric measures reflect the average of three 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 min of rest with a standard manual mercury sphygmomanometer (Diamond Deluxe Industrial Electronics and Products, Pune, India) and stethoscope (3M Littmann, 3M India Ltd., Bangalore, India) by auscultatory method [39]. 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 [40]. 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-h urine sample, in space of at least 6 months apart [36] 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) [41]. The patients were divided into two groups depending on the absence or presence of nephropathy in the form of microalbuminuria (AER of 30–299 mg/day) or macroalbuminuria (AER $\geq$ 300 mg/day) [1, 36]. Patients were considered cases if the AER was $\geq$ 30 mg/day in at least two of the three 24-h collections [42]; all others were considered as controls.

Sampling and Biochemical Analysis

After an overnight fast of 12 h, 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 method, CV%: 3.4) [43], cholesterol (Cholesterol oxidase method, CV%: 3.9) [44], triglycerides (Enzymatic method, CV%: 3.6) [45], HDL-C (Phosphotungstic method, CV%: 4.7) [46], LDL-C (CV%: 3.6) [47], HbA1c (Immunoturbidimetric method, CV%: 3.9) [48] and creatinine (modified Jaffe's method, CV%: 2.5) [49] 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 (Bangalore, India) based on standard proteinase K technique [50] and then either stored at -20°C or amplified immediately. All DNA samples were amplified within 3 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: $0.020\ \mu\text{g/ml/cm}$) [51] in a standard spectrophotometer (UV–Vis double beam—2205, Systronics, Ahmedabad, India).

DNA Analysis: Amplification and Detection of ACE Gene I/D Polymorphism

Two oligonucleotide primers, forward: 5'-CTG GAG AGC CAC TCC CAT CCT TTC T-3' and reverse: 5'-GGG ACG TGG CCA TCA CAT TCG TCA G-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) [52]. 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, 1 U thermus aquaticus DNA polymerase, 1.5 mM magnesium chloride, amplification buffer contained 20 mM Tris–hydrochloric acid and 50 mM KCl. The thermocycling profile consisted of 1 min of initial denaturation at 94 °C followed by 30 cycles of amplification of denaturation at 95 °C for 30 s, 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. Thus, 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') [53] 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 [53]. 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, IL, USA). Data were expressed as mean $\pm$ 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>) [54] 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 estimated 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

During the study period 82 patients were lost to follow-up. Of the remaining 309 T2DM patients, 159 (55.97 % males) belonged to Ahir community and 150 (52.66 % males) were of Rabari community. Table 1 shows the subgroup and clinical characteristics of the study participants. The mean reported duration of diabetes mellitus in the Ahir participants were 13.4 ± 3.1 , 14.6 ± 3.9 and 13.8 ± 3.4 years in groups with normo-, micro-, and macroalbuminuria, respectively and there were no significant differences in age ($p \geq 0.068$), duration of the disease ($p \geq 0.084$), total body fat percentage ($p \geq 0.237$), BMI ($p \geq 0.062$), waist-to-hip ratio ($p \geq 0.579$), prevalence of hypertension ($p \geq 0.243$) and sex distribution between groups ($p \geq 0.052$). Neither systolic/diastolic BP nor lipid profile parameters were different between the three patient groups ($p \geq 0.052$), and this was attributed to comparable number of patients who were receiving antihypertensive and lipid lowering drugs ($p \geq 0.66$) in the groups analysed. However, detailed documentation of antidiabetic, antihypertensive and lipid lowering medication could not be obtained for all patients and hence these data are not reported. Nephropathy patients had a significantly higher levels of HbA1c ($p \leq 0.034$), together with higher plasma creatinine ($p \leq 0.038$) compared to controls, and more severe albuminuria was significantly associated with higher levels of plasma creatinine ($p < 0.05$). In patients of Rabari community, the results of above mentioned variables and pattern were similar to those of Ahir participants (Table 1).

Genotype distribution in T2DM patients with nephropathy and T2DM patients without nephropathy were significantly different in each community (Ahir: χ^2 :8.87, 2 d.f. $p = 0.019$; Rabari: χ^2 :11.01, 2 d.f. $p = 0.004$). The difference was due to significantly higher frequency of ACE D/D homozygotes [(Ahir: 42.46 vs. 22.09 %, OR = 2.603, $p = 0.007$) (Rabari: 45.71 vs. 21.25 %, OR = 3.121, $p = 0.002$)], and lower frequency of heterozygote ACE I/D [(Ahir: 36.98 vs. 58.13 %, OR = 0.423, $p = 0.008$) (Rabari: 35.71 vs. 58.75 %, OR = 0.39, $p = 0.005$)] (Table 2). This pattern of association was similar in cases {irrespective of the extent of DN (micro- and macroalbuminuria)}; {Ahir: (Microalbuminuric patients— χ^2 :6.51, 2 d.f. $p = 0.039$; Macroalbuminuric patients— χ^2 :5.63, 2 d.f. $p = 0.04$);

Table 1 Characteristics of enrolled participants

Parameters	Ahir community		Rabari community	
	Control (n:86)		Control (n:80)	
	Normoalbuminuria (n:86)	Microalbuminuria (n:32)	Normoalbuminuria (n:80)	Microalbuminuria (n:34)
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Age (years)	56.60 $\pm$ 7.55	54.71 $\pm$ 8.11	52.44 $\pm$ 7.46	54.29 $\pm$ 6.18
Sex, males; n (%)	47 (54.65 %)	18 (56.25 %)	43 (53.75 %)	17 (50.00)
Active smokers, n (%)	14 (16.27 %)	06 (18.75 %)	16 (20.00 %)	05 (14.70 %)
Duration of DM (years)	13.4 $\pm$ 3.1	14.6 $\pm$ 3.9	13.7 $\pm$ 3.4	15.1 $\pm$ 4.7
Prevalence of HTN, n (%)	54 (62.79 %)	23 (71.87 %)	53 (66.25 %)	25 (73.52 %)
Creatinine (mg/dl)	0.88 $\pm$ 0.28	1.03 $\pm$ 0.48 [†]	0.85 $\pm$ 0.26	1.01 $\pm$ 0.41 [†]
BMI (kg/m ²)	28.66 $\pm$ 3.01	29.20 $\pm$ 4.83	29.37 $\pm$ 3.13	30.03 $\pm$ 4.12
SBP (mmHg)	134.32 $\pm$ 14.55	139.82 $\pm$ 11.94	135.20 $\pm$ 10.66	137.34 $\pm$ 13.87
DBP (mmHg)	84.03 $\pm$ 5.69	85.93 $\pm$ 6.14	82.14 $\pm$ 6.02	84.29 $\pm$ 6.38
HbA1C (%)	8.29 $\pm$ 0.44	8.51 $\pm$ 0.62 [†]	8.32 $\pm$ 0.45	8.55 $\pm$ 0.56 [†]
TG (mg/dl)	157.89 $\pm$ 14.54	163.12 $\pm$ 13.60	166.23 $\pm$ 16.43	169.07 $\pm$ 19.22
Total Cholesterol (mg/dl)	217.93 $\pm$ 20.48	223.48 $\pm$ 18.60	213.36 $\pm$ 19.05	219.95 $\pm$ 20.72
HDL-C (mg/dl)	43.81 $\pm$ 3.12	42.54 $\pm$ 4.88	42.19 $\pm$ 3.94	41.90 $\pm$ 3.87
LDL-C (mg/dl)	118.22 $\pm$ 14.37	124.31 $\pm$ 16.57	122.39 $\pm$ 14.36	126.72 $\pm$ 14.68
T-C/HDL	4.99 $\pm$ 0.52	5.09 $\pm$ 0.46	5.01 $\pm$ 0.78	5.27 $\pm$ 0.89
LDL/HDL	2.71 $\pm$ 0.54	2.93 $\pm$ 0.67	2.86 $\pm$ 0.71	3.03 $\pm$ 0.86
Waist circumference (cm)	98.77 $\pm$ 10.90	99.14 $\pm$ 11.60	96.11 $\pm$ 9.87	98.54 $\pm$ 9.66
Hip circumference (cm)	105.23 $\pm$ 9.11	103.40 $\pm$ 9.09	104.33 $\pm$ 8.81	105.40 $\pm$ 9.44
Total body fat %	32.49 $\pm$ 6.01	31.22 $\pm$ 6.10	30.01 $\pm$ 6.11	32.44 $\pm$ 5.90

* $p < 0.01$ (macro vs. normo)[†] $p < 0.05$ (micro vs. normo)[‡] $p < 0.05$ (macro vs. micro)[§] $p < 0.05$ (macro vs. normo)

Table 2 Genotype distribution and allele frequencies of ACE I/D polymorphism in T2DM patients with nephropathy (cases) and T2DM patients without nephropathy (controls)

Genotypes	Control (n:86)	Cases (n:73)	p	OR	OR (95 % CI)	
					Lower bound	Upper bound
Ahir community						
II	17 (19.76 %)	15 (20.54 %)	0.903	1.05	0.483	2.283
ID	50 (58.13 %)	27 (36.98 %)	0.008	0.423	0.223	0.801
DD	19 (22.09 %)	31 (42.46 %)	0.007	2.603	1.307	5.185
Expected frequencies from HW equation	20.51 II (23.84 %), 42.98 ID (49.97 %) & 22.51 DD (26.17 %)	11.13 II (15.24 %), 34.75 ID (47.60 %) & 27.13 DD (37.16 %)				
HWE: χ^2 ; p	2.297; 0.130	3.628; 0.057				
p allele (I) frequency	0.49	0.39				
q allele (D) frequency	0.51	0.61				
Genotypes						
Genotypes	Control (n:80)	Cases (n:70)	p	OR	OR (95 % CI)	
					Lower bound	Upper bound
Rabari community						
II	16 (20.0 %)	13 (18.57 %)	0.897	0.793	0.397	2.023
ID	47 (58.75 %)	25 (35.71 %)	0.005	0.39	0.201	0.756
DD	17 (21.25 %)	32 (45.71 %)	0.002	3.121	1.53	6.365
Expected frequencies from HW equation	19.5 II (24.37 %), 39.99 ID (49.98 %), & 20.5 DD (25.62 %)	9.29 II (13.27 %), 32.42 ID (46.31 %) & 28.29 DD (40.41 %)				
HWE: χ^2 ; p	2.455; 0.117	3.668; 0.055				
p allele (I) frequency	0.49	0.36				
q allele (D) frequency	0.51	0.64				

Genotypes	Microalbuminuria (n:32)				Macroalbuminuria (n:41)						
	n	p	OR	95 % CI	n	p	OR	95 % CI			
									Lower bound	Upper bound	Upper bound
Ahir community											
II	7 (21.87 %)	0.8	1.137	0.421	3.965	8 (19.51 %)	0.973	0.984	0.386	2.512	
ID	11 (34.37 %)	0.023	0.377	0.162	0.879	16 (39.02 %)	0.046	0.461	0.216	0.985	
DD	14 (43.75 %)	0.022	2.743	1.155	6.511	17 (41.46 %)	0.026	2.498	1.118	5.578	
Expected frequencies from HW equation	4.88 II (15.25 %), 15.23 ID (47.59 %) & 11.88 DD (37.12 %)					6.24 II (15.21 %), 19.51 ID (47.58 %) & 15.24 DD (37.17 %)					
HWE: χ^2 ; p	2.472; 0.1159					1.328; 0.2491					
p allele (I) frequency	0.39					0.39					
q allele (D) frequency	0.61					0.61					
Genotypes											
Microalbuminuria (n:34)				Macroalbuminuria (n:36)							
n	p	OR	95 % CI	Lower bound	Upper bound	n	p	OR	95 % CI	Upper bound	Lower bound
Rabari community											
II	6 (17.64 %)	0.771	0.857	0.304	2.420	7 (19.44 %)	0.945	0.966	0.359	2.6	
ID	13 (38.23 %)	0.047	0.435	0.191	0.989	12 (33.33 %)	0.013	0.351	0.154	0.8	
DD	15 (44.11 %)	0.015	2.926	1.234	6.937	17 (47.22 %)	0.006	3.316	1.423	7.724	
Expected frequencies from HW equation	4.6 II (13.52 %), 15.81 ID (46.5 %) & 13.6 DD (40 %)					4.69 II (13.02 %), 16.61 ID (46.13 %) & 14.69 DD (40.80 %)					
HWE: χ^2 ; p	1.076; 0.3002					2.774; 0.0958					
p allele (I) frequency	0.37					0.36					
q allele (D) frequency	0.63					0.64					

Rabari: (Microalbuminuric patients— χ^2 :6.42, 2 d.f. $p = 0.04$; Macroalbuminuric patients— χ^2 :8.87, 2 d.f. $p = 0.01$) (Table 3). Albeit the genotype frequency resulted in a higher frequency of the D allele in the group of cases (total or subgroup) than controls, the difference in allele frequency did not reach statistical significance with this sample size ($p \geq 0.06$). Testing genetic equilibrium between the observed and expected genotypes using HWE showed ACE genetic variants were confirming to the law in all groups (Table 2, 3).

In univariate analysis, the ACE D/D homozygotes was significantly associated with diabetic nephropathy {Ahir: $p = 0.007$; OR [95 % CI] = 2.603 [1.307–5.185]; Rabari: $p = 0.002$; OR [95 % CI] = 3.121 [1.53–6.365] (Table 2)}, implying a risk of approximately 2.5 and 3 times higher than the other genotypes in Ahir and Rabari community respectively. Even after categorizing cases on the basis of albumin excretion into micro- and macroalbuminuria the pattern of association remain similar (Table 3).

To determine independent correlates of the susceptibility and severity of albuminuria, variables with statistically significant associations with albuminuria in the univariate analysis (creatinine, HbA1c, ACE genotype) as well as age, sex, diabetes duration, systolic/diastolic BP (as clinically significant variables) were included in a multivariate analysis. In the model, the DD genotype was a significant and the strongest independent predictor of microalbuminuria

(Ahir: $p = 0.0362$, OR = 2.65, 95 % CI 1.89–6.36; Rabari: $p = 0.024$, OR = 2.81, 95 % CI 1.9–6.65), thus confirming the association of ACE I/D polymorphism with development of nephropathy, after adjustment for the co-variables. However, it did not independently change the odds of having macroalbuminuria versus microalbuminuria (Table 4), thereby demonstrating that ACE genotype are of value in predicting DN development rather than DN severity, but the ID genotype did not show a significant association ($p \geq 0.34$). The other variable(s) with significant, independent association with microalbuminuria were HbA1c (Ahir: $p = 0.0421$; Rabari: $p = 0.033$) and creatinine (Ahir: $p = 0.0484$; Rabari: $p = 0.031$).

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%)] in each of the community {(For micoralbuminuria: Ahir: χ^2 :5.171 OR = 2.74, $p = 0.023$; Rabari: χ^2 :5.922 OR = 2.93, $p = 0.015$) (For macroalbuminuria: Ahir: χ^2 :4.966 OR = 2.50, $p = 0.026$; Rabari: χ^2 :7.783 OR = 3.32, $p = 0.005$) (Table 5)}.

Discussion

In this study, we tested association of ACE gene I/D polymorphism with DN using a case–control approach in

Table 4 Comparison between the groups by multivariate regression analysis

Covariate	Microalbuminuria versus normoalbuminuria			Macroalbuminuria versus microalbuminuria		
	<i>p</i>	OR	95 % CI	<i>p</i>	OR	95 % CI
Ahir community						
Age	0.32	1.024	0.986–1.065	0.780	1.016	0.643–1.033
Sex	0.721	0.912	0.344–1.576	0.686	0.813	0.311–1.542
Diabetes duration	0.098	1.58	0.88–2.79	0.31	1.15	0.81–1.98
SBP	0.0825	1.69	0.95–2.47	0.754	1.015	0.86–2.34
DBP	0.46	1.021	0.75–1.17	0.785	1.011	0.66–1.15
HbA1c	0.0421	2.13	0.76–2.65	0.752	1.014	0.66–1.021
Creatinine	0.0484	2.01	0.72–2.35	0.092	1.49	0.83–2.19
DD versus other genotype	0.0362	2.65	1.89–6.36	0.838	0.91	0.3367–2.184
ID versus other genotype	0.34	1.18	0.86–1.68	0.5790	0.829	0.3039–1.998
Rabari community						
Age	0.29	1.028	0.991–1.058	0.217	1.088	0.783–1.1477
Sex	0.741	0.946	0.412–1.61	0.697	0.822	0.379–1.613
Diabetes duration	0.081	1.62	0.75–2.38	0.314	1.15	0.87–1.52
SBP	0.35	1.14	0.77–1.65	0.426	1.022	0.78–1.64
DBP	0.0836	1.61	0.89–2.27	0.811	1.012	0.69–1.12
HbA1c	0.033	2.25	0.71–3.05	0.582	1.038	0.66–1.48
Creatinine	0.031	2.31	0.57–3.56	0.097	1.51	0.86–2.24
DD versus other genotype	0.024	2.81	1.9–6.65	0.673	0.96	0.432–2.04
ID versus other genotype	0.48	1.16	0.75–1.44	0.713	0.79	0.322–1.78

Table 5 Analysis of genetic risk factors under dominant, co-dominant and recessive mode

	Ahir community			Rabari community		
	Dominant mode [(DD% + ID%) vs. II%]	Co-dominant mode [ID% vs. (II% + DD%)]	Recessive mode [DD% vs. (II% + ID%)]	Dominant mode [(DD% + ID%)vs. II%]	Co-dominant mode [ID % vs. (II% + DD%)]	Recessive mode [DD% vs. (II% + ID%)]
Microalbuminuria versus normoalbuminuria						
<i>p</i>	0.801	0.169	0.023	0.769	0.069	0.015
OR	0.88	1.52	2.74	1.17	1.75	2.93
95 % CI	0.33–2.37	0.83–2.78	1.16–6.51	0.41–3.29	0.95–3.23	1.23–6.94
Macroalbuminuria versus normoalbuminuria						
<i>p</i>	0.973	0.130	0.026	0.945	0.055	0.005
OR	1.02	1.53	2.50	1.04	1.77	3.32
95 % CI	0.40–2.59	0.88–2.66	1.12–5.58	0.38–2.79	0.98–3.20	1.42–7.72
Macroalbuminuria versus microalbuminuria						
<i>p</i>	0.804	0.997	0.845	0.847	0.942	0.079
OR	1.15	1.00	0.91	0.89	1.02	1.13
95 % CI	0.37–3.61	0.55–1.84	0.36–2.32	0.27–2.97	0.55–1.9	0.44–2.91

two different communities (Ahir and Rabari) from Kutch region, Gujarat (India). We analysed the genotypic and the allelic distributions of the ACE I/D polymorphism between normoalbuminuric controls and DN patients. Genotype data were available for all participants, and in each community, frequency of DD genotype were found to be significantly higher in cases {irrespective of the extent of DN (micro- and macroalbuminuria)} than in control participants ($p < 0.05$). Therefore, we suggest that the genetic variation at the ACE locus as D/D variant in intron 16, contribute to an increased risk of nephropathy in T2DM patients, but not extent of DN severity (as the allelic or genotypic distribution was comparable between the two DN groups) in studied population. Our findings were in conformity with other studies [26–31] but not all [32, 55, 56]. This difference may possibly be due to different races, methods of quantitation and patient selection; as proteinuria in adults is a multifactorial condition frequently linked with diabetes, the issue of whether proteinuria is due to diabetes or some other etiology remains debatable, but there was no uncertainty in our group of patients on the role of T2DM in diabetic nephropathy constitution as we excluded the patients who had proteinuria/renal disorders before their diabetes was diagnosed, thus we confined our study to diabetic kidney diseases.

The potential effects of ACE gene polymorphism was viewed from two perspectives, firstly, susceptibility to DN and secondly, severity of DN. Since this is an observational-analytical-retrospective study, in order to derive a more comprehensive estimation, we employed two strategies: To assess the association of ACE I/D polymorphism and DN susceptibility, we compared normoalbuminuric patients with microalbuminuric patients, whereas to address the

second prospective, patients with macroalbuminuria were compared with microalbuminuria. In the present study, we had the advantage that the three groups of patients were closely matched in a long list of potentially confounding variables (Table 1), which made it easier to compare the extent to which each subject was previously exposed to the variable of interest. Although longer T2DM duration was seen in the DN patients versus normoalbuminuric patients (with macroalbuminuric patients having lower disease duration than microalbuminuric patients), it did not attained statistical significance ($p = 0.052$), conflicting with previous findings [57–59] that longer T2DM duration increases the risk of DN development. However, in our study univariate analysis revealed that in addition to ACE genotype, significantly higher HbA1c [59–61], and creatinine levels [58, 59, 61] were associated with renal injury, in accord with other studies on DN development.

To assess the independent role of studied variables as a potential determinant, we employed multivariate regression, in which we included age, sex, B.P. and diabetes duration (as clinically significant variables) along with variables with significant associations in univariate analysis (creatinine, HbA1c, ACE genotype) in the covariate list of regression analysis. Although the cases and controls in our study were similar in age, sex, B.P. and diabetes duration, we considered these variables as potential confounders in multivariate analysis to prevent missing any significant, confounding effects. Our results showed that the effect of the DD genotype persisted even when controlling in multivariate models for known covariates that are associated with microalbumin excretion (Table 4).

To the best of our knowledge, this is the first report of the association between the three ACE polymorphic

variants and DN in an ethnically homogeneous population from Kutch region, and the first to identify that the DD genotype (vs. other genotype) independently increased the risk of nephropathy in diabetes (2.65-fold in Ahir and 2.81-fold in Rabari, Table 4), while the ID genotype did not alter the risk significantly. This pattern suggests a recessive mode of inheritance in allele D of the ACE gene polymorphism and was substantiated by analysis of genetic risk factors under dominant, co-dominant and recessive model (Table 5). Thus, the contribution of ACE gene polymorphism to the heritable part of risk for diabetic nephropathy seems important, and is supported by findings from published meta-analyses [28, 62] along with other studies done among Indians [31, 63], Japanese [29], French [64], Egyptian [65] and Taiwan [27] diabetic patients. However, our results do not coincide with studies done among Chinese [66], Tunisian [55], French [56], Turkish [34] and Iranian [67] diabetic cases. This discrepancy in polymorphisms association studies may be reconciled by several factors, with ethnic/racial and geographic differences being a potential reason i.e. it is possible that the potential genetic effects on risk of complex diseases are different across various ethnic populations; which is further exemplified by D allele frequency of at around 0.6 in Ahir & Rabari DN patients respectively (Tables 2, 3), corresponding with reports among Europeans [24], Tunisian [68] and Caucasians [69] but lower values were reported in Asians (0.38) [70, 71], Chinese [72] and Pima Indians (0.29) [73], suggesting an ethnic difference. Therefore, the difference between the significant association among one population and null effects among other may not be surprising. Hence, we confirm that ethnic differences should be accounted for when studying the ACE gene. However, it has also been suggested that the main effects of the individual loci might be too small to be observed and that loci may contribute to the complex diseases only by their interaction with other genes [74]. Thus, it is possible that ACE I/D polymorphism affects the outcome through a linkage disequilibrium with other causative genes or polymorphisms, due to which, positive association will be found in populations with tighter linkage but not in populations with weaker linkage.

In this respect, to clarify the significance of this gene polymorphism, we recommend a large-scale prospective cohort study and should preferentially focus on gene–gene and gene–environment interactions as well as haplotype analyses which may eventually provide a better, comprehensive understanding of the association between the ACE I/D polymorphism and nephropathy risk. In the interim, our analysis indicated that genetic variation at the ACE locus as D/D variant, contribute to an increased risk of nephropathy in T2DM patients but not extent of DN severity and thus can be used as genetic marker for the susceptibility to DN.

Nonetheless, this study has few limitations. Firstly, the study involves a small sample size; still results of our study are particularly relevant, as our trial fulfils the criteria of a good genetic association study suggested by Hattersley et al. [75]. Secondly, the design was cross-sectional and therefore cannot provide causal relationship, for which longitudinal studies are required. 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 and finally, lack of treatment details (use, duration of ACE inhibitors and their dosages) of the patients is a minor limitation as treatment with ACE inhibitors or ARBs does not influence genetic polymorphisms. In spite of these limitations, our study provides more definitive support for the role of ACE polymorphisms in diabetic nephropathy than the many previous cross-sectional studies and the results provide important information for clinical practice, which have implications for the care of patients with diabetes.

References

1. Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogensen CE, Parving HH, et al. American Diabetes Association. Nephropathy in diabetes. *Diabetes Care*. 2004;27(Suppl 1): 79–83.
2. Williams ME. Diabetic CKD/ESRD 2010: a progress report. *Semin Dial*. 2010;23:129–33.
3. Agius E, Attard G, Shakespeare L, Clark P, Vidya MA, Hattersley AT, et al. Familial factors in diabetic nephropathy: an offspring study. *Diabet Med*. 2006;23:331–4.
4. Gohda T, Tanimoto M, Watanabe-Yamada K, Matsumoto M, Kaneko S, Hagiwara S, et al. Genetic susceptibility to type 2 diabetic nephropathy in human and animal models. *Nephrology (Carlton)*. 2005;10(Suppl 2):22–5.
5. Vijay V, Snehalatha C, Sinha K, Lalitha S, Ramachandran A. Familial aggregation of diabetic kidney disease in type 2 diabetes in south India. *Diabetes Res Clin Pract*. 1999;43:167–71.
6. Kanakamani J, Ammini AC, Gupta N, Dwivedi SN. Prevalence of microalbuminuria among patients with type 2 diabetes mellitus—a hospital based study from north India. *Diabetes Technol Ther*. 2010;12:161–6.
7. Debnath S, Thameem F, Alves T, Nolen J, Al-Shahrouri H, Bansal S, et al. Diabetic nephropathy among Mexican Americans. *Clin Nephrol*. 2012;77:332–44.
8. ESRD Incidence Study Group, Stewart JH, McCredie MR, Williams SM. Geographic, ethnic, age-related and temporal variation in the incidence of end-stage renal disease in Europe, Canada and the Asia-Pacific region, 1998–2002. *Nephrol Dial Transpl*. 2006;21:2178–83.
9. Young BA, Maynard C, Boyko EJ. Racial differences in diabetic nephropathy, cardiovascular disease, and mortality in a national population of veterans. *Diabetes Care*. 2003;26:2392–9.
10. Hostetter TH, Troy JL, Brenner BM. Glomerular hemodynamics in experimental diabetes mellitus. *Kidney Int*. 1981;19:410–5.

11. Ezzidi I, Mtiraoui N, Kacem M, Chaieb M, Mahjoub T, Almawi WY. Identification of specific angiotensin-converting enzyme variants and haplotypes that confer risk and protection against type 2 diabetic nephropathy. *Diabetes Metab Res Rev*. 2009;25:717–24.
12. Osawa N, Koya D, Araki S, Uzu T, Tsunoda T, Kashiwagi A, et al. Combinational effect of genes for the renin–angiotensin system in conferring susceptibility to diabetic nephropathy. *J Hum Genet*. 2007;52:143–51.
13. Prasad P, Tiwari AK, Kumar KM, Ammini AC, Gupta A, Gupta R, et al. Chronic renal insufficiency among Asian Indians with type 2 diabetes: I. role of RAAS gene polymorphisms. *BMC Med Genet*. 2006;7:42–50.
14. Ballerman BJ, Zeidel ML, Gunning ME, Brenner BM. Vasoactive peptides and the kidney. In: Brenner BM, Rector FC, editors. *The kidney*. 4th ed. Philadelphia: Saunders; 1991. p. 510–83.
15. Ruiz-Ortega M, Gómez-Garre D, Alcázar R, Palacios I, Bustos C, González S, et al. Involvement of angiotensin II and endothelin in matrix protein production and renal sclerosis. *J Hypertens*. 1994;12:S51–8.
16. Burns KD. Angiotensin II and its receptors in the diabetic kidney. *Am J Kidney Dis*. 2000;36:449–67.
17. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev*. 2000;52:11–34.
18. Thomas GN, Tomlinson B. Prevention of macrovascular disease in type 2 diabetic patients: blockade of the renin–angiotensin–aldosterone system. *Curr Diabetes Rev*. 2008;4:63–78.
19. Berl T. Renal protection by inhibition of the renin–angiotensin–aldosterone system. *J Renin Angiotensin Aldosterone Syst*. 2009;10:1–8.
20. Mehdi UF, Adams-Huet B, Raskin P, Vega GL, Toto RD. Addition of angiotensin receptor blockade or mineralocorticoid antagonism to maximal angiotensin-converting enzyme inhibition in diabetic nephropathy. *J Am Soc Nephrol*. 2009;20:2641–50.
21. Hubert C, Houot AM, Corvol P, Soubrier F. Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. *J Biol Chem*. 1991;266:15377–83.
22. Mattei MG, Hubert C, Alhenc-Gelas F, Roeckel N, Corvol P, Soubrier F. Angiotensin converting enzyme gene is on chromosome 17. *Cytogenet Cell Genet*. 1989;51:1041.
23. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest*. 1990;86:1343–6.
24. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, et al. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet*. 1992;51:197–205.
25. Crisan D, Carr J. Angiotensin I-converting enzyme: geno-type and disease associations. *J Mol Diagn*. 2000;2:105–15.
26. Ahluwalia TS, Ahuja M, Rai TS, Kohli HS, Bhansali A, Sud K, et al. ACE variants interact with the RAS pathway to confer risk and protection against type 2 diabetic nephropathy. *DNA Cell Biol*. 2009;28:141–50.
27. Tien KJ, Hsiao JY, Hsu SC, Liang HT, Lin SR, Chen HC, et al. Gender-dependent effect of ACE I/D and AGT M235T polymorphisms on the progression of urinary albumin excretion in Taiwanese with type 2 diabetes. *Am J Nephrol*. 2009;29:299–308.
28. Wang F, Fang Q, Yu N, Zhao D, Zhang Y, Wang J, et al. Association between genetic polymorphism of the angiotensin-converting enzyme and diabetic nephropathy: a meta-analysis comprising 26,580 subjects. *J Renin Angiotensin Aldosterone Syst*. 2012;13:161–74.
29. Okuno S, Utsugi T, Ohno T, Ohyama Y, Uchiyama T, Tomono S, et al. Angiotensin-converting enzyme gene polymorphism as a potent risk factor for developing microalbuminuria in Japanese patients with type 2 diabetes mellitus: a 9-year follow-up study. *J Int Med Res*. 2003;31:290–8.
30. Ortega-Pierres LE, Gómez García A, Rodríguez-Ayala E, Figueroa-Núñez B, Figueroa-Núñez VM, Higareda-Mendoza AE, et al. Angiotensin I converting enzyme insertion/deletion gene polymorphism in a Mexican population with diabetic nephropathy. *Med Clin (Barc)*. 2007;129:6–10.
31. Movva S, Alluri RV, Komandur S, Vattam K, Eppa K, Mukkavali KK, et al. Relationship of angiotensin-converting enzyme gene polymorphism with nephropathy associated with type 2 diabetes mellitus in Asian Indians. *J Diabetes Complicat*. 2007;21:237–41.
32. Jayapalan JJ, Muniandy S, Chan SP. Null association between ACE gene I/D polymorphism and diabetic nephropathy among multiethnic Malaysian subjects. *Indian J Hum Genet*. 2010;16:78–86.
33. Eroglu Z, Cetinkalp S, Erdogan M, Kosova B, Karadeniz M, Kutukculer A, et al. Association of the angiotensinogen M235T and angiotensin-converting enzyme insertion/deletion gene polymorphisms in Turkish type 2 diabetic patients with and without nephropathy. *J Diabetes Complicat*. 2008;22:186–90.
34. Arzu Ergen H, Hatemi H, Agachan B, Camlica H, Isbir T. Angiotensin-I converting enzyme gene polymorphism in Turkish type 2 diabetic patients. *Exp Mol Med*. 2004;36:345–50.
35. Declaration of Helsinki. Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects. 52nd World Medical Assembly, Edinburgh, October, 2000.
36. Mauer M, Fioretto P, Woredek Y, Friedman EA, Gottschalk RW. Diabetic nephropathy. In: Schrier CW, editor. *Diseases of the kidney*. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 2083–128.
37. Laakso M, Pyorala K. Age of onset and type of diabetes. *Diabetes Care*. 1985;8:114–7.
38. World health Organization Expert Consultation. Appropriate body mass index for Asian populations and its implications for policy and intervention strategies. *Lancet*. 2004;363:157–63.
39. Rose GA, Blackburn H. Cardiovascular survey methods. WHO Monogr Series. 1968;56:90–5.
40. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. *JAMA*. 2003;289:2560–72.
41. Medcalf E, Newman DJ, Gorman EG, Price CP. Rapid, robust method for measuring low concentrations of albumin in urine. *Clin Chem*. 1990;36:446–9.
42. Myers GL, Miller WG, Coresh J, Fleming J, Greenberg N, Greene T, et al. National Kidney Disease Education Program Laboratory Working Group. Recommendations for improving serum creatinine measurement: a report from the Laboratory Working Group of the National Kidney Disease Education Program. *Clin Chem*. 2006;52:5–18.
43. Trinder P. Determination of blood glucose using an oxidase–peroxidase system with a non-carcinogenic chromogen. *J Clin Pathol*. 1969;22:158–61.
44. Allain CC, Poon LS, Chan CSG, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem*. 1974;20:470–5.
45. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem*. 1982;28:2077–80.
46. Burstein M, Morfin R. Precipitation of alpha lipoproteins in serum by sodium phosphotungstate in the presence of magnesium chloride. *Life Sci*. 1969;8:345–8.

47. Assmann G, Jabs HU, Kohnert U, Nolte W, Schriewer H. LDL-cholesterol determination in blood serum following precipitation of LDL with polyvinyl sulfate. *Clin Chim Acta*. 1984;140:77–83.
48. Metus P, Ruzzante N, Bonvicini P, Meneghetti M, Zaninotto M, Plebani M. Immunoturbidimetric assay of glycated hemoglobin. *J Clin Lab Anal*. 1999;13:5–8.
49. Chasson AL, Grady HJ, Stanley MA. Determination of creatinine by means of automatic chemical analysis. *Tech Bull Regist Med Technol*. 1960;30:207–12.
50. Jeanpierre M. A rapid method for the purification of DNA from blood. *Nucleic Acids Res*. 1987;15(22):9611.
51. Tataurov AV, You Y, Owczarzy R. Predicting ultraviolet spectrum of single stranded and double stranded deoxyribonucleic acids. *Biophys Chem*. 2008;133:66–70.
52. Rigat B, Hubert C, Corvol P, Soubrier F. PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). *Nucleic Acids Res*. 1992;20:1433–6.
53. Lindpaintner K, Pfeiffer MA, Kreutz R, Stampfer MJ, Grodstein F, LaMotte F, et al. A prospective evaluation of an angiotensin converting enzyme gene polymorphism and the risk of ischemic heart disease. *New Engl J Med*. 1995;332:706–11.
54. Rodriguez S, Gaunt TR, Day IN. Hardy–Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. *Am J Epidemiol*. 2009;169(4):505–14.
55. Arfa I, Abid A, Nouira S, Elloumi-Zghal H, Malouche D, Mannai I, et al. Lack of association between the angiotensin-converting enzyme gene (I/D) polymorphism and diabetic nephropathy in Tunisian type 2 diabetic patients. *J Renin Angiotensin Aldosterone Syst*. 2008;9:32–6.
56. Hadjadj S, Gallois Y, Alhenc-Gelas F, Chatellier G, Marre M, Genes N, et al. Angiotensin-I-converting enzyme insertion/deletion polymorphism and high urinary albumin concentration in French type 2 diabetes patients. *Diabet Med*. 2003;20:677–82.
57. Al-Salman RA, Al-Basri HA, Al-Sayyad AS, Hearnshaw HM. Prevalence and risk factors of albuminuria in type 2 diabetes in Bahrain. *J Endocrinol Invest*. 2009;32:746–51.
58. Cardoso CR, Salles GF. Predictors of development and progression of microvascular complications in a cohort of Brazilian type 2 diabetic patients. *J Diabetes Complicat*. 2008;22:164–70.
59. Mtiraoui N, Ezzidi I, Turki A, Chaieb M, Mahjoub T, Almawi WY. Renin–angiotensin–aldosterone system genotypes and haplotypes affect the susceptibility to nephropathy in type 2 diabetes patients. *J Renin Angiotensin Aldosterone Syst*. 2011;12:572–80.
60. Stolar M. Glycemic control and complications in type 2 diabetes mellitus. *Am J Med*. 2010;123(Suppl 3):3–11.
61. Tu ST, Chang SJ, Chen JF, Tien KJ, Hsiao JY, Chen HC, et al. Prevention of diabetic nephropathy by tight target control in an Asian population with type 2 diabetes mellitus: a 4-year prospective analysis. *Arch Intern Med*. 2010;170:155–61.
62. Ng DP, Tai BC, Koh D, Tan KW, Chia KS. Angiotensin-I converting enzyme insertion/deletion polymorphism and its association with diabetic nephropathy: a meta analysis of studies reported between 1994 and 2004 and comprising 14,727 subjects. *Diabetologia*. 2005;48:1008–16.
63. Naresh VV, Reddy AL, Sivaramakrishna G, Sharma PV, Vardhan RV, Kumar VS. Angiotensin converting enzyme gene polymorphism in type II diabetics with nephropathy. *Indian J Nephrol*. 2009;19:145–8.
64. Hadjadj S, Belloum R, Bouhanick B, Gallois Y, Guilloteau G, Chatellier G, et al. Prognostic value of angiotensin I converting enzyme I/D polymorphism for nephropathy in type 1 diabetes mellitus: a prospective study. *J Am Soc Nephrol*. 2001;12:541–9.
65. El-Baz R, Settin A, Ismaeel A, Khaleel AA, Abbas T, Tolba W, et al. MTHFR C677T, A1298C and ACE I/D polymorphisms as risk factors for diabetic nephropathy among type 2 diabetic patients. *J Renin Angiotensin Aldosterone Syst*. 2012;13:472–7.
66. Wong TY, Chan JC, Poon E, Li PK. Lack of association of angiotensin-converting enzyme (DD/II) and angiotensinogen M235T gene polymorphism with renal function among Chinese patients with type II diabetes. *Am J Kidney Dis*. 1999;33:1064–70.
67. Felehgari V, Rahimi Z, Mozafari H, Vaisi-Raygani A. ACE gene polymorphism and serum ACE activity in Iranians type II diabetic patients with macroalbuminuria. *Mol Cell Biochem*. 2011;346:23–30.
68. Rebai M, Kharrat N, Ayadi I, Rebai A. Haplotype structure of five SNPs within the ACE gene in the Tunisian population. *Ann Hum Biol*. 2006;33:319–29.
69. Solini A, Vestra M, Saller A, Nosadini R, Crepaldi G, Fioretto P. The angiotensin-converting enzyme DD genotype is associated with glomerulopathy lesions in type 2 diabetes. *Diabetes*. 2002;51:251–5.
70. Hsieh MC, Lin SR, Hsieh TJ, Hsu CH, Chen HC, Shin SJ, et al. Increased frequency of angiotensin-converting enzyme DD genotype in patients with type 2 diabetes in Taiwan. *Nephrol Dial Transpl*. 2000;15:1008–13.
71. Feng Y, Niu T, Xu X, Chen C, Li Q, Qian R, et al. Insertion/deletion polymorphism of the ACE gene is associated with type 2 diabetes. *Diabetes*. 2002;51:1986–8.
72. Lee EJ. Population genetics of the angiotensin-converting enzyme in Chinese. *Br J Clin Pharmacol*. 1994;37:212–4.
73. Foy CA, McCormack LJ, Knowler WC, Barrett JH, Catto A, Grant PJ. The angiotensin-I converting enzyme (ACE) gene I/D polymorphism and ACE levels in Pima Indians. *J Med Genet*. 1996;33:336–7.
74. Culverhouse R, Suarez BK, Lin J, Reich T. A perspective on epistasis: limits of models displaying no main effect. *Am J Hum Genet*. 2002;70:461–71.
75. Hattersley AT, McCarthy MI. What makes a good genetic association study? *Lancet*. 2005;366:1315–23.