



Levels of β 2-Microglobulin and Association of ACE Gene I/D Polymorphism with Type 2 Diabetes Mellitus Patients

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Abstract

Background

Diabetes mellitus is a metabolic ailment which is an outcome of defect in insulin secretion, insulin action, or both. The ACE gene systematizes Angiotensin Converting Enzyme (ACE) and is the most profoundly studied gene because of its crucial role in the renin-angiotensin system (RAS). The insertion/ deletion (I/D) of a 287bp long Alu repetitive sequence in intron 16 is responsible for three genotypes, DD and II homozygotes and ID heterozygotes. ACE levels differ in carriers with respect to their genotype; DD carriers have twice the levels of ACE than that found in II genotype individuals. The main purpose of this study is to investigate the association of ACE gene I/D polymorphism in Pakistani Type 2 Diabetes Mellitus (T2DM) patients primarily from Lahore.

Methods: Hundred patients (T2DM) and fifty healthy controls were enrolled in this study. The ACE I/D polymorphism, located in intron 16, was analyzed by a triple primer method called nested-PCR and subsequently the results were analyzed by gel electrophoresis. Urine samples were also collected from patients suffering from diabetic nephropathy for the determination of beta-2 microglobulin.

Results: The frequency of ACE genotypes DD, ID and II among the patients with type 2 diabetes mellitus was found to be 76%, 10%, 14% whereas in control subjects, 38%, 4%, 8% respectively. Other clinical parameters like blood sugar level and body mass index were also evaluated to find an association with genotype. The findings showed a non-significant association of ACE genotype with Blood Sugar Level (BSL) and Body Mass Index (BMI). Of the hundred T2DM patients enrolled in the study ten (10%) were of diabetic nephropathy. Beta-2-microglobulin was measured in the urine of these patients as well as healthy controls. A significant association between β ₂-microglobulin and T2DM was found by Fischer's exact test. Serum creatinine values were noted and correlation was found. The results showed a positive correlation but non-significant between creatinine and beta-2-microglobulin.

Conclusion: In the present study, no significant association was found between ACE gene I/D polymorphism and T2DM. Gender of the T2DM patients showed null effect on genotype. Other clinical parameters like blood sugar level and body mass index also revealed a non-significant association with the ACE gene genotype.

Keywords: Type 2 diabetes mellitus; Angiotensin Converting Enzyme; blood sugar level; body mass index.

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Background:

Diabetes mellitus is a metabolic ailment which is an outcome of defect in insulin secretion, insulin action, or both [1]. Diabetes is ubiquitous and has evolved into a routine global health problem that affects more than 170 million people worldwide. Diabetes mellitus leads to a heterogeneous group of disorders involving chronic hyperglycemia which involves disturbed carbohydrate metabolism, fat and protein metabolism. This is a consequence of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues; especially muscle and liver [2]. The ACE gene systematizes Angiotensin Converting Enzyme and is most profoundly studied gene because of its crucial role in the renin-angiotensin system (RAS). ACE catalyzes the conversion of inactive decapeptide Angiotensin I into active octapeptide Angiotensin II. Angiotensin II is a vasoactive, aldosterone-stimulating peptide, and causes mediation of cell growth, proliferation and induction of endothelial dysfunction [3]. ACE enzyme has a crucial role in mammals as it plays a pivotal role in the maintenance of homeostatic mechanism thus maintaining blood pressure through vasoconstriction and electrolyte balance of blood by being a stimulant of the hormone aldosterone [4]. It is also responsible for the inactivation of bradykinin [5], further it is found to play a role in the signal transduction mechanism [6]. ACE is a mammalian enzyme encoded by the ACE gene, which has been cloned and sequenced. The gene is 21kb, positioned on the long arm of chromosome 17(17q23) and is composed of 26 exons and 25 introns [9]. Numerous studies have been executed to determine the association of ACE I/D polymorphism on the onset of diabetes mellitus. Now we know that the ACE levels are under genetic control most studies focused on an insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene as a marker for a functional polymorphism. The results are conflicting as some studies support this statement considering ACE I/D polymorphism as a factor responsible for diabetes mellitus whereas other researches oppose it. Studies done on South Indian patients revealed positive association between the D allele (ID and DD genotype) of the ACE polymorphism and diabetic proteinuria in South Indian type 2 diabetic patients [10]. Studies done in Gujrat-India indicate a substantial association of ACE I/D polymorphism and type 2 diabetes at least in South Asian and Southwest Asian populations. As ACE participates in the metabolic and haemodynamic functions and thus the authors suggested that ACE I/D polymorphism might be involved in the pathogenesis of T2DM particularly in the Asian population [7]. The main objective of this study is to evaluate the association of ACE insertion/deletion I/D polymorphism with type 2 Diabetes mellitus as well as to measure the levels of beta-2 microglobulin only in type 2 Diabetes mellitus patients overlapping with diabetic nephropathy.

Methods

Study design

A total of 100 blood samples were obtained from type 2 diabetes patients coming to JAIDE, Jinnah Hospital, Lahore. Fifty unrelated subjects without the history of type 2 diabetes were randomly selected from general population to serve as control subjects for the association studies. An informed written consent was taken from all the subjects recruited in the study as well as from the matched controls. Five ml of the blood was collected in tubes containing ethylene diamine tetra acetic acid (EDTA) as whole blood for the extraction of DNA. Urine samples were taken from patients suffering from diabetic nephropathy for the analysis of β -2 microglobulin.

DNA isolation and Genetic analysis

DNA isolation was carried out by manual genomic DNA extraction method [24]. The ACE I/D polymorphism, located in intron 16, was analyzed by a triple primer method with a nested-PCR primer situated completely within the insertion sequence of I allele. The inclusion of a third PCR primer is the most reliable strategy for ACE genotyping. The PCR reaction was performed in a total volume of 50 μ l using 250 ng of genomic DNA, 100 pmol of each of the primer mixture and 2 X PCR mixture. ACE gene was amplified with the reported primers [11]. Nested PCR conditions consisted of an initial 95°C denaturation for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 sec, annealing step at 53°C, elongation at 72°C for 5 minutes (Figure-2.12). The DNA fragments were analyzed on 2% agarose gel. The banding patterns of the 3 possible genotypes were as follows: DD: 210bp fragment; II: 498 and 264 bp fragments; ID: 498, 264 and 210 bp fragments.

Beta-2-microglobulin:

Beta-2-microglobulin was measured in urine by using Orgentec kit (Cat # ORG 5BM). Highly purified anti-human-beta-2-microglobulin antibodies are bound to microwells. The reaction is based on indirect enzyme immunoassay (ELISA) method with these steps: beta-2-Microglobulin present in a patient sample binds to the antibody coated forming an antigen-antibody complex. Washing of the microwells removes unbound unspecific components. During incubation with anti-beta-2-microglobulin enzyme-conjugate immunologically a conjugate/antibody/antigen complex is formed. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue colour. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow colour is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of antibodies present in the original sample.



Statistical analysis

All the statistical analysis was carried out by using SPSS version 13.0. Distribution of genotype frequencies between type 2 diabetes mellitus patients and control was compared using chi-square test. Relationship between clinical variables (body mass index and blood glucose level) and ACE genotype was compared with one-way analysis of variance (ANOVA).

Results and Discussion

Of the 100 patients, fifty-nine (59%) were females and forty-one (41%) were males. The female versus male ratio was 1.4:1. Of the 100 patients, ninety-nine were married and 1 was unmarried. Mean age of type 2 diabetic patients at the time of study was 49.96 ± 10.38 years (Range: 30-70 years).

Association of ACE genotype with BSL and BMI of type 2 diabetic patients

Random blood sugar level (BSL) of all the patients coming to the hospital was measured by a glucometer and values were noted. Other body measurement like height was measured in inches for each subject in a standing upright position. Weight of each subject was measured in kilograms without shoes and in light clothing. Body mass index (BMI) was calculated as weight in kilograms over height in meters squared. Overweight was defined as $BMI \geq 24$ [13]. Analysis of Variance (ANOVA) was carried out by SPSS ver 13 in order to check whether or not the BSL and BMI are associated to the ACE genotype (Table 1 & 2).

Distribution of ACE genotypes

In our study we found a non-significant association and the level of significance was at $p \geq 0.05$.

The frequencies of ACE DD, ID and II genotypes among type 2 diabetic patients were 76 (76%), 10 (10%) and 14 (14%) and in 50 control subjects 38 (76%), 10 (20%) and 2 (4%) respectively. Null effect of gender on ACE (I/D) polymorphism was found by applying Chi-square test in the cases with the respective controls (Table 3 & 4).

Levels of beta-2 –microglobulin in diabetic nephropathic patients and creatinine correlation

Of the 100 T2DM patients, 10 were the cases of diabetic nephropathy that were characterized by increased beta-2-microglobulin (B2M) and high creatinine levels in the urine. The creatinine values of the patients were noted by checking the latest laboratory test records. Serum B2M was measured in the urine quantitatively by ELISA. Of the 10 diabetic nephropathy patients six patients were negative while 4 were positive for B2M. The controls were found to be 100% negative for B2M. Fischer's exact test was applied as the expected frequencies were small (p -value < 0.05). Thus, a significant association was found between B2M and diabetic nephropathy. Higher levels of B2M were seen in these diabetic nephropathic patients. Accuracy matrix was applied to calculate the positive predictive value (PPV) which was calculated to be 100 (39-100), negative predictive value (NPV) 62.5 (35-84), specificity 100 (39-100) and sensitivity 40 (12-73) of Beta-2-Microglobulin (Cat # ORG 5BM) kit. Correlation was evaluated between creatinine and beta-2-microglobulin to find the effect of both on each other. The p -value was (< 0.05) and it was found the beta-2 microglobulin and creatinine were not associated with each other as shown in (Figure 2).

Healthy people with DD genotype have high Angiotensin II levels as compared to those with that II genotype [12]. High ACE enzyme activity is associated with high Angiotensin II level and low bradykinin level. The renin-angiotensin system also exist in skeletal muscle where it modifies the use of substrate through a kallikrein-kinin system, where low ACE levels lead to increased glucose uptake during exercise [14]. Low ACE activity has been reported to increase sensitivity to insulin, storage of glycogen, skeletal-muscle glucose uptake, and glucose transport. The local adipose Renin-Angiotensin System might change mobilization of substrate from fat stores [15]. It has also been reported to increase suppression of insulin of non-esterified fatty-acid flux [16]. Altogether, the findings show that low ACE levels are beneficial to glucose metabolism. In 1990 Rigat and coworkers were the first to identify ACE gene I/D polymorphism. The findings of their study showed that the activity of circulating ACE depends on the insertion/deletion (I/D) polymorphism [18]. In the present study carried out on 100 T2DM patients; 59 were females while remaining 41 were males. The female to male ratio was 1.4:1 with females outnumbering the male patients with T2DM. The mean age of patients was 49.96 ± 10.38 years. Regarding the marital status 99 patients were married and 1 was unmarried. In 1999 Hawthorne *et al.*, conducted a study in UK on Pakistani type 2 diabetics and found that female patients need to be educated more to eliminate the disease [34]. The frequency of T2DM was highest between the age group 41-50 years. In 2008 van-Valkengoedet *et al.*, studied ACE gene (I/D) polymorphism and glycemic state in a huge population. The results showed null effect of ACE I/D polymorphism on glycemic status of patients with diabetes mellitus [35]. In the present study, statistical analysis was done to evaluate the association of ACE gene genotype and blood sugar level of the subjects involved in the study. The results showed null effect of genotype on the glycemic status of the patients. All the patients enrolled in this study both male and female showed no effect of genotype on their glycemic status. Studies done by Vibert *et al.*, 1981 suggested that beta-2-microglobulin concentrations is an easy and precise method of finding minor degrees of renal damage and checking the outcomes of medication [36]. Of the hundred T2DM patients enrolled in the study ten were of diabetic nephropathy. Beta-2-microglobulin was measured in the urine of these patients as well as healthy controls and a significant association was found by Fischer's exact test. Serum creatinine values were noted and correlation was found. The results showed a positive correlation but not significant between creatinine and beta-2-microglobulin.



Sikdaret *et al.*, 2013 studied the occurrence of ACE gene polymorphism in 91 Bengali individuals. The distribution pattern of D allele frequency did not differ significantly in patients having diabetic nephropathy, diabetic without nephropathy and control subjects. However, the authors suggested that ACE gene, I/D polymorphism is an appropriate marker for studying genetic variation among various human populations. Also, ACE gene is stable in nature representing a distinctive evolutionary event [20]. Similarly, in this study ACE gene I/D polymorphism was selected to evaluate its association with T2DM. ACE is a key enzyme of RAS, moreover high angiotensin II level and low bradykinin level in (RAS) associated with insulin-resistance and considered risk factors for T2DM [33]. A study was conducted at Sardjito hospital, Indonesia by Sinorita *et al.*, in 2010 to evaluate the frequency of ACE gene I/D polymorphism in T2DM patients. A non-significant association of metabolic syndrome, the components of metabolic syndrome and variants of ACE gene in T2DM patients was found. Similarly, Jayapalan *et al.*, (2010) conducted a study to evaluate ACE gene I/D polymorphism in T2DM subjects in multiethnic Asian population. Similar genotype frequencies were found in healthy controls and T2DM patients showing a non-significant association of ACE gene with T2DM. Ethnicity and gender also showed null effect of ACE I/D polymorphism on T2DM [32]. The results of the present study were similar to the findings of Jayapalan *et al.*, (2010) showing a non-significant association of ACE I/D polymorphism with T2DM. Moreover, the study also supports the findings of present study showing null effect of gender on ACE gene I/D polymorphism. In 2009 Baroudi *et al.*, evaluated ACE gene I/D polymorphism in Jerba Island, Tunisia among two ethnic populations in Tunisia. They also found that DD genotype is involved in the pathogenesis of T2DM in Jerbian population. Moreover, Baroudi *et al.*, (2009) also emphasized on the vital role of ethnicity, which should be considered an important factor in this genetic studies of T2DM [22]. In a cohort study of 10.2 years Conen *et al.*, 2007 revealed that 24,309 Caucasian women free from diabetes at baseline failed to show any association of ACE genotype with diabetes [23]. Eroglu *et al.*, (2008) examined the polymorphism in ACE gene in Turkish T2DM patients with and without nephropathy. The findings demonstrated no significant variation in the frequencies of ACE (I/D) polymorphism between T2DM patients with or without diabetic nephropathy. Also, the ACE gene does not play any part with the risk of developing diabetic nephropathy. The findings of Ergolu *et al.*, (2008) support results of the present study which showed a lack of association of ACE I/D polymorphism with T2DM. The first study in Turkey was conducted by Ergen *et al.*, (2004) to examine the effect of ACE gene I/D polymorphism in T2DM patients. In this study, a non-significant association between the allelic frequencies and the presence of neuropathy, retinopathy and cardiac events in patients with and without nephropathy were found. However, higher frequency of DD genotype was found in diabetic patients than controls [21]. Similarly, Yang *et al.*, (2006) investigated the association between ACE I/D polymorphism and T2DM in Han Chinese population. The individuals with DD genotype showed a significant association and increased susceptibility to T2DM. Nevertheless, the Yang *et al.*, (2006) did not suggest any mechanisms involved in the association of DD genotype with T2DM. Unlike Yang *et al.*, (2006) the present study showed the same frequency of DD genotype in both the patients and controls [31]. The frequency of ACE genotype between T2DM patients with or without nephropathy was determined by Arfa *et al.*, 2008 in the Tunisian population. The outcome of Arfa *et al.*, (2008) study is somewhat similar to this study as the results reflect no relation of ACE gene I/D polymorphism with diabetic nephropathy or with T2DM [30]. Ramachandran *et al.*, 2008 studied ACE I/D polymorphism in hypertensive and T2DM subjects in Malaysian population. In this study they found a strong association of DD genotype and D allele in hypertensive and T2DM patients. However, the author did not find any association with respect to gender or ethnicity [29]. Bhavani *et al.*, 2005 conducted a study to evaluate the association of I/D polymorphism with hypertension or diabetes and its role in enhancing the susceptibility to diabetes and/or hypertension. It was seen that D allele of ACE gene protects against diabetes, nevertheless it increases susceptibility to hypertension specifically when associated with T2DM [27]. Feng *et al.*, in a case-control study examined ACE gene I/D polymorphism in T2DM. In this study population, the DD genotype was higher in the cases than in control group indicating association of DD genotype with increased susceptibility to T2DM [28].

Conclusion

Thus, it is concluded that T2DM is due to complex interaction of genetic and environmental factors which is found to be fatal when overlapped with various complications. ACE I/D polymorphism was found to be actively involved in the disease. However, no significant association was found between ACE gene I/D polymorphism and T2DM. This could be due to limitation because of the small study population. Lahore population represents a genetically relatively homogenous population due to inbreeding for centuries. Consanguineous marriages are common so there are chances that genomic structure of local population may be unique. However, there is need to study a large sample size to characterize Pakistani population with T2DM to show some association of ACE gene I/D polymorphism.

Competing interests

The authors declare no competing interests

Authors' contributions

Both the authors have contributed equally.

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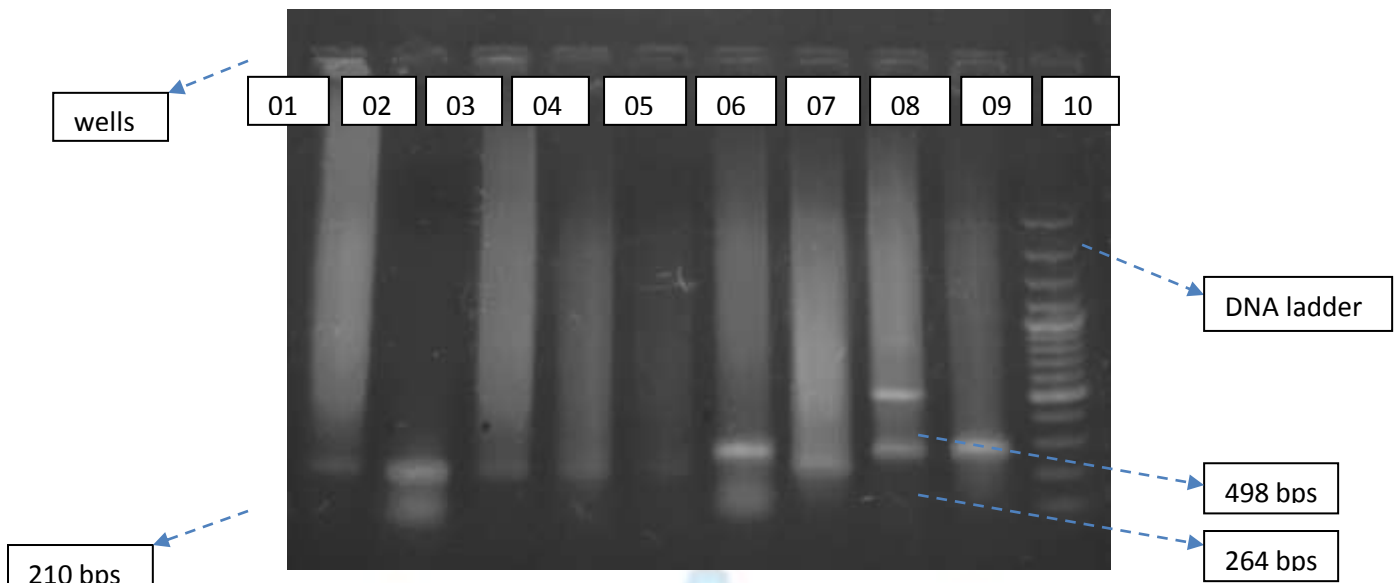


Figure 1: Detection of ACE insertion/deletion polymorphism with nested PCR.

Well 10 represents the low range ladder, well 1,2,3,4,5,6,7 and 9 represents the band of 210bp so the genotype is DD. Well 8 represents the bands of 264bp and 498bp indicating II genotype

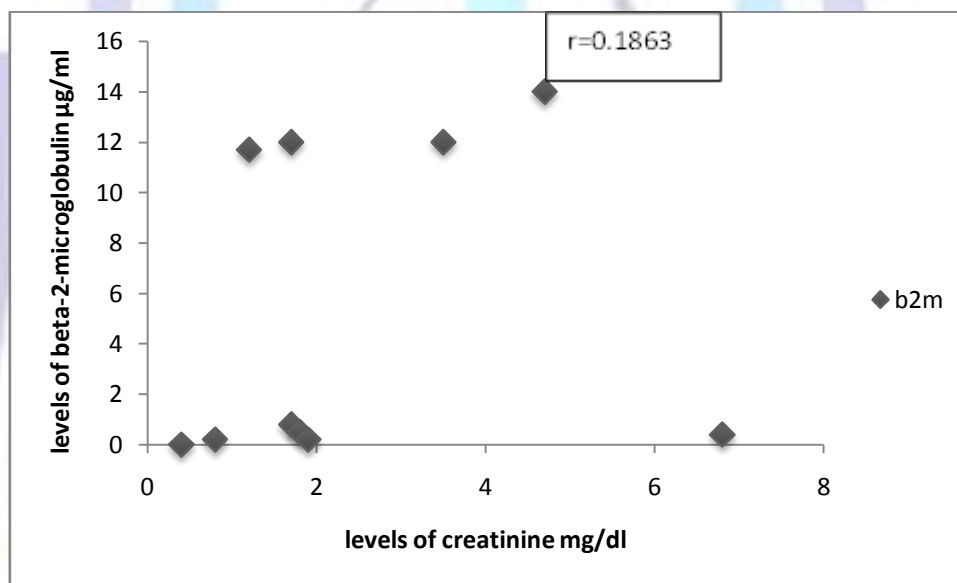


Figure 2: Correlation between levels of creatinine and beta-2-microglobulin

Table 1: Association of BMI and Genotype

BMI (Body mass index) (kg/m ²) in Genotypes	Mean	F-value	P-value
DD	25.2795± 5.74734	1.434	0.243
II	23.6043±3.14559		
ID	22.7730±2.38368		



Table 2: Association of Random BSL and Genotype

BSL (Blood sugar level) (mg/dl) in Genotypes	Mean	F-value	P-value
DD	271.9737±115.47271	0.400	0.671
II	286.5714±134.01706		
ID	243.0000±122.88929		

Table 3: Chi-square test of the association of ACE gene (I/D) polymorphism in T2DM patients and controls

Patient Status	DD	II	ID	Results
Cases (n = 100)	76	14	10	p =0.368
Controls (n = 50)	38	210		
Total	114	18	18	

Table 4: Chi-square test of the effect of gender on the association of ACE gene (I/D) polymorphism in T2DM patients and controls

Patient Status		DD	II	ID	Results
Cases (n = 100)	male	32	6	3	p =0.756
	Female	44	8	7	
Controls (n = 50)	male	28	4	7	
	Female	13	0	1	