Serum Ace and Ace I/D Polymorphism: Risk of Type 2 Diabetes with and Without Nephropathy in Pakistani Cohort

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Abstract

Objective: To find serum ACE (Angiotensin converting enzyme) level and the role of ACE I/D polymorphism with T2DM (Type 2 Diabetes Mellitus) among the Pakistani cohort.

Methods: A total of 110 diagnosed T2DM patients and 100 normotensive healthy controls with an age range of 35-65 years were randomly selected for this study. All cases were screened for the T2DM based on random and fasting blood sugar levels and confirmed by HbA1c test. Genomic DNA was extracted from peripheral blood samples by the Salting out method and ACE I/D polymorphism was genotyped using insertion-deletion polymorphism and serum level of ACE was also determined. All statistical analysis was conducted using SPSS version 16 and all values are significant at a p-value less than 0.05. Hardy Weinberg Equilibrium (HWE) was calculated for any deviation of allele frequencies from predicted. The Chi-square test was used to evaluate the association between ACE polymorphism and the risk of diabetes. The odds ratio along with a 95% confidence interval via binary logistics regression analysis was used to find out the diabetic risk associated with ACE genotyping.

Results: The analysis showed higher ACE levels among T2DM patients with nephropathy (mean= 158 ± 38.9) and without nephropathy (mean= 128.23 ± 46.8) as compared to controls (mean= 94.4 ± 28.6). No significant association (2 = 7.402, p-value=0.116) was observed between ACE genotyping and T2DM. However, those who have DD (O.R= 2.714, 95% CI=0.943-7.809) genotype of ACE polymorphism were at risk of diabetes but the results were non-significant. However, no risk was present at the diabetic nephropathy females.

Conclusion: These outcomes propose that the ACE gene may not contribute to T2DM in the Pakistani cohort. However, ACE levels were higher among T2DM with and without nephropathy patients.

Keywords: Genetic Polymorphism, Association, T2 DM, Diabetic Nephropathy

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Introduction

Diabetes mellitus (DM) is a universal public health problem with multiple complications and an increasing prevalence. According to International Diabetes Federation (IDF) diabetes atlas 2021, Pakistan is ranked 3 out of 215 countries in the world, having 33 million cases of DM at an age between 20 and 79 years[1]. It has many forms depending on the cause among which type 2 Diabetes (T2D M) is most common and makes it the major prob lem that affects Pakistani's life[2]. Characteristics of T2DM include insulin resistance, and metabolic disturbance of carbohydrates,proteins and lipids (1). T2DM is influenced by both inherent and environmental factors[3]. Diabetic kidney disease (DKD) is one of the serious microvascular complications of diabetes mellitus. About 20–40% of diabetic patients suffer from DKD,which is the leading cause of end-stage renal disease (ESRD).

The renin-angiotensin system (RAS) is involved in most of the pathological processes that lead to DKD. Angiotensin-I converting enzyme (ACE), a zi-

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nc metallopeptidase which converts angiotensin-I into peptide angiotensin-II to excite aldosterone and inactivate bradykinin. ACE is assumed to perform other physiological roles because of its wide distribution and enzymatic specificity. ACE is a membranebound enzyme present in endothelial cells, epithelial cells as well as biological fluids. The mechanism by which the circulating ACE is synthesized is still obscure, However, the structure of circulating ACE resembles the cellular ACE. The elevated level of circulating ACE is mostly observed in sarcoidosis, that's why its elevated levels are widely used in the diagnosis and therapeutic aspects of the disease[4].

Genetics is not only a risk factor but it plays a devastating role in the development of diabetes. Approximately 60 genes are involved in the regulation of insulin action, insulin level and adipose metabolism and polymorphisms of these genes are also identified which linked with diabetes. Among which ACE insertion deletion (ACE I/D) polymorphism is one of them. The ACE I/D gene polymorphism is a prominent ACE gene sequence variation which employs its diverse influence on the tissue expression, circulating ACE activity; and angiotensin-II production[5,6]. ACE gene is characterized by the insertion and deletion of 287bp fragment (Alu repeated) in intron 16 of the gene. Presence of the D allele increases whereas presence of I allele generally lowers the stability of the ACE mRNA transcript and ACE levels in blood[7].So, altered ACE level can take a part in pathogenesis of T2DM. A study observed 'sex-specific' association among women between ACE 'I/D' polymorphism and T2DM whereas no influence observed among men[8]. Other study done on the South Indian regional population showed that DD genotype and the D allele of the ACE gene can be a risk factor for T2DM[9]. Present study is designed to investigate the association between ACE polymorphism and risk of diabetes in Pakistani female population with ACE genotype.

Material and Methods

The present study is a cross-sectional,single-center study. This study was conducted from Jan 2016 to Dec 2016 in the Department of Physiology, University of Karachi with approval number (DRC-KU-236/2012) according to the guidelines of the Helsinki declaration.The diabetic samples were collected from diabetic O.P.D. in Sindh government hospital New Karachi whereas controls were selected randomly by door-to-door survey in Karachi and others were taken from general O.P.D.in same hospital. The sample size was calculated using the online sample size calculator OpenEpi (https://www.openepi.com/Menu/OE_Menu.htm) with 80% of statistical power and 95% of two-sided confidence level. (210) female subjects (110 diagnosed females and 100 normotensive healthy females) of 35-65 years of age were app-roached for this study. All cases were clinically diagnosed based on random and fasting blood sugar levels and confirmed by HbA1c test. Diabetic nephropathy was diagnosed by routine urine al-bumin/creatinine ratio testing and GFR and confirmed by repeated elevation in urinary albumin level. However,the controls did not have any clinically diagnosed disease. All subjects were given to fill out a written consent in local language before sample collection. Demographic, history and anthropometry were taken using a self-structured ques-tionnaire.

Serum levels of glucose, creatinine, albumin and ACE were analyzed using commercial kits. BMI was calculated using standard formula: BMI = weight (in kg) / height in (meter)^2. According to the World health Organization and National Institute of Health (NIH), BMI is classified as Un-derweight, Normal weight, Overweight and Obese among Asians and South Asians[10].

DNA was extracted from leukocytes according to "salting out" method. The basic principles are R.B.Cs washing, lysis of leukocytes, protein digestion by Proteinase K, precipitation of protein by NaCl, Removal of RNA by adding RNAs and stored extracted DNA at -80 ^{0}C. ACE gene was amplified using a pair of sense and antisense primers 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-GATGGTGGCCATCACATTCGTCAGAT-3'. PCR mixture was prepared in a reaction volume of 10µl. This mix-

ture contain 5µl of 2X green master mix (Promega, USA) was added with 0.2 µl of each 20 pmol primer given below in table and 0.3 µl of genomic DNA while rest of volume was made up by nuclease free water. Genomic DNA is amplified in an automated thermal cycler (veriti^{TM},applied Biosystems,USA) in following PCR conditions: Initial Denaturation 94^0C for 1min, Denaturation 94^0C for 1 min, Annealing 58^0C for 1min, Extension 72^0C and Final Extension 72^0C.

Results of PCR were separated by electrophoresis on 1.5 % agarose gel containing 0.45 g of agarose in 30ml of IX TBE stained with 0.5µg/ml of ethidium bromide, then visualized through chemi-DOC –It2 (UVP, UK) using vision work LS software (version 7.1). The PCR product is a 190 bp fragment in the deletion (D allele) and a 490 bp fragment in the presence of Insertion (I allele).

All data management and statistical analysis was conducted using SPSS 16 and all values are taken significant at P<0.05.The Chi square test was used to evaluate the association between ACE gene polymorphisms. Hardy-Weinberg equilibrium (HWE) was calculated for any deviation of allele frequencies from predicted among controls using goodness of fit model. Odds ratio along with 95-% confidence intervals via logistic regression ana-lysis was used to find out the diabetic risk asso-ciated with ACE genotype.

Results

The characteristics of all study participants were given in Table 1.

Table 1. Characteristics of Studied Participants

Characteristics	Cases n=110	Controls n=100
Age (years):		
31- 40	28 (24.54%)	27 (27%)
41-50	52 (49.09%)	49 (49%)
51-60	25 (21.8%)	21 (21%)
61-70	5 (4.5%)	3 (3%)
Ethnicity:		
Urdu speaking	90 (81%)	85 (85%)
Pathan	13 (11.8%)	8 (8%)
Punjabi	5 (4.5%)	5 (5%)
Others	2 (1.8%)	2 (2%)
Marital status:		
Married:	104 (94.5%)	98 (99%)
Single:	6 (5.4%)	2 (1%)
No. of pregnancies:		
0-5	66 (63.4%)	55 (56%)
6-11	39 (37.5%)	42 (43%)
More than 11	5 (4.8%)	1 (1%)
Gestational diabetes:		
Yes	5 (4.8%)	2 (2%)
No	99 (95.2%)	96 (98%)
Family history:		
Diabetes	55 (50%)	21 (21%)
Hypertension	4 (3.6%)	18 (18%)
Cardiovascular disease	6 (5.4%)	14 (14%)
Pregnancy related problems	2 (1.8%)	1 (1%)
Physical activity:		
Sedentary	109 (99%)	99 (99%)
Active	1 (1%)	1 (1%)
B.M.I		
Under weight	3 (2%)	2 (2%)
Overweight	33 (34%)	32 (32%)
Obese	38 (34%)	19 (19%)
Normal	36 (32%)	47 (47%)

Biochemical profile shows higher Fasting and Random glucose, Urinary albumin and ACE levels in T2DM with and without nephropathy as compared to controls (Table 2). Increase ACE levels may be associated with the insulin resistance.

Table 2. Comparison of biochemical profile between cases and controls

Biochemical profile	Controls (n=100)	T2DM without nephropathy (n=54)	T2DM with nephropathy (n=56)
Fasting blood glucose glucose (mg/dl)	92.4 ± 84	248 ± 54.2	295 ± 62.8
Random glucose (mg/dl)	106 ± 6.2	178 ± 23.2	221 ± 40.1
Urinary Creatinine (g/l)	1.02 ± 0.25	1.18 ± 0.46	0.88 ± 0.45
Urinary Albumin (mg/l)	4.6 ± 2.2	15.31 ± 7.7	82.8 ± 44.7
Serum ACE levels (ng/ml)	94.4 ± 28.6	128.23 ± 46.8	158 ± 38.9

The size of amplified products of ACE I/D gene DD variant is 190bp and II is 490bp long. The expected genotype frequencies were calculated for healthy controls, and Hardy-Weinberg equilibrium was calculated based on the goodness-of-fit X^2 statistics. According to Hardy-Weinberg equilibrium, the ACE genotype were not in Hardy-Weinberg equilibrium and significantly deviated (X^2 = 11.789, P-value=0.00059).

The genotype distribution and allele frequencies of T2DM females with and without nephropathy and controls are shown in Table 3. No significant association (÷^{2} = 7.402, p-value=0.116) was observed among diabetic and normal females with ACE genotype. However, those who have DD (O.R= 2.7-14,95% CI= 0.943-7.809) genotype of ACE polymorphism were at odds of having diabetes but results were non-significant. However, no risk was present in the diabetic nephropathy females (Table 3).

Table 3. Association of ACE gene polymorphism and type 2 diabetes with and without nephropathy

Geno type	Controls n (%)	T2DM with nephropathy n (%)	T2DM without nephropathy n (%)	Pearson's Chi2* (p-value)	T2DM with nephropathy O.R (95% CI)	T2DM without nephropathy O.R (95% CI)
II	19 (19)	10 (19)	9 (15)		Reference	Reference
DI	67 (67)	34 (66)	33 (55)	7.402 (0.116)	0.964 (0.404 - 2.301)	1.04 (0.424 - 2.547)
DD	14 (14)	7 (13)	18 (3)		0.95	2.714
Total	100	51	60		(0.29 - 3.114)	(0.943 - 7.809)
Allele						
I	105 (52)	54 (52)	51 (43)	3.773	1	1
D	95 (48)	48 (47)	69 (57)	(0.152)	1.002 (0.622 - 1.616)	1.526 (0.967 - 2.408)
Total	200	102	120			

Analysis Values in parenthesis are percentages
P-value<0.05 = significant difference
Odd ratio with 95% confidence intervals via binary logistic regression

Discussion

The present study examined the association between insertion, deletion, and polymorphism of the Angiotensin-converting enzyme gene and T2DM with and without nephropathy and the odds of having diabetes. The study also investigated the comparison of serum ACE levels among cases and controls. Results showed that ACE levels were higher in T2DM with and without nephropathy as compared to controls.Increased ACE level increases Angiotensin level and these involve pathophysiological changes such as hemodynamic effects of angiotensin II contribute increase vasoconstriction and reduction in perfusion of skeletal muscles and ultimately glucose utilization. A study reported that Ang II increases insulin resistance by lowering glucose utilization by downregulation of GLUT 1 in the cell membrane and disarrangement of actin filaments[11]. Local RAAS mechanism is also found in the pancreas or especially in beta cells, so an increase in Angiotensin II levels decreases blood flow in the pancreas, alters insulin secretion, and plays role in insulin resistance[12].

According to our findings, ACE genotyping deviates significantly in accordance with the Hardy principle, which is similar to a previous study conducted on Tunisian population[13]. Deviation in Hardy principle in genotype distribution of ACE could be due to certain factors including increased chances of misclassification of genotype, unpredicted assortment in ethnicity, intermarriages in families that relate to breeding, non-random coupling, and emigration. This study also observed that the DD genotype increases the risk of diabetes in the studied population though non-significantly. It was also reported in a recent study that ACE I/D polymorphism increases the risk of diabetes among cardiovascular patients in women of Faisalabad, Pakistan[14]. In this study, a slightly high frequency of the D allele in cases as compared to control did not show significant differences, while the DD genotype increases the risk of diabetes, but non-significant results were obtained. Evident association of ACE I/D polymorphism with T2DM was present among the Iraqi population[15]. A recent study concluded that the ACE DD variant has a strong association with diabetic nephropathy but is not a risk factor for the development of disease[16].

A meta-analysis also reported that the D allele increases the risk of T2DM by influences and altered glucose metabolism[17]. A systemic review also reported that a variant of RAAS mechanism gene increases the risk of diabetes[18].

The present investigation has the limitation of a relatively small sample size along with the specific gender. These limitations could be improved by enrolling a large number of individuals of both genders.

Conclusion

ACE gene may not be associated with the risk of diabetes among Pakistani population.This study provided information for further research because diabetes is multifactorial and polygenetic disorder and exact single known cause of diabetes is remained unknown and prevalence of diabetes in Pakistani population is increasing with the time. ACE gene may not be associated with the odds of having T2DM with and without nephropathy among Pakistani population.

Conflict of Interest

Authors have no conflict of interest and no grant/funding from any organization.

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