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Original Research Article

Association of angiotensin converting enzyme gene polymorphism with diabetic nephropathy in patients using lisinopril

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ABSTRACT

Angiotensin Converting Enzyme (ACE) plays an important role in the development and progression of Diabetic nephropathy (DN). The present study was designed to determine the possible association between ACE gene polymorphism and DN. The study included 242 samples: DN (n = 121), type 2 Diabetes mellitus (DM2) (n = 60) and control (n = 61). The blood samples were collected from the subjects, followed by DNA extraction. Insertion deletion polymorphism of ACE gene studied using specific primers. Patients using Lisinopril were followed for two months. The ACE genotype distribution in DN patients was as follows: DD (n = 47; 38.84%), II (n = 17; 14.04%) and DI (n = 57; 47.10%). In DM group the genotype distribution was DD (n = 4; 6.66%), II (n = 25; 41.66%) and DI (n = 31; 51.66%) while in control group DD (n = 38; 62.29%), II (n = 1; 1.63%) and DI (n = 22; 36.06%). The comparison of II genotype to DD genotype was reflected by p-value =0.0001, OR=17.28 and 95% CI 5.313-49.58. The percent decrease of micro-albuminuria after two months with the use of Lisinopril 10 mg twice a day in DD, II and DI genotype of DN were 31.27%, 12.37% and 16.81%, respectively. Our findings revealed that DD genotype has strong association with DN but not a risk factor for development of disease.

1. Introduction

Type 2 Diabetes mellitus (DM2) is a multifactorial metabolic disorder leading to loss of glycemic homeostasis in the body.[1] According to an International Diabetes Federation (IDF) 415 million peoples are affected with DM2 world widely and is expected to be 642 million by the year 2040.[2] DM may progress to serious complications if left untreated, which can affect patients' quality of life and reduce life expectancy.[3] The complications include macrovascular complications (coronary artery disease, peripheral artery disease and stroke) or microvascular complications (diabetic nephropathy, retinopathy, and neuropathy).[4,5] Diabetic nephropathy (DN) in early stage can be identified by persistent micro-albuminuria[6] which can be defined as the increased excretion of albumin in urine in the range of 20-200 μg/min or when the amount of albumin is 30-300mg/24 h.[7] Globally one third of diabetic patients are facing with the complication of DN,[8] while Rossing and de Zeeuw reported that about 39% of diabetic patients are struggling with micro-albuminuria worldwide,[9]

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DN pathogenesis is multifactorial by involving both genetic and environmental factors.[10] Angiotensin II in DN has been proposed to provoke renal vasoconstriction, mesangial cell contraction and increased pressure in glomerular capillary.[11] Genetic polymorphism in Renin angiotensin system may alter the pathogenesis of DN[12] Gene responsible for ACE in human is found on long arm of chromosome 17q23, and there is 287 base pair I/D polymorphism in intron 16.[13] The ACE gene is 21 kilo bases (kb) long and consists of 25 introns and 26 exons[14]. Patients with deletion homozygous (DD) genotype are known to produce increased levels of ACE enzyme and thus are more prone to DN,[15] while patients with insertion homozygous (II) genotype are producing less amount of ACE and are genetically protected.[16] Deletion insertion heterozygous (DI) genotypic are intermediate of aforementioned genotypes.[16] Studies have reported that individuals with D allele are at high risk for DN as compared to II genotype.[17] ACE inhibitors and angiotensin II receptor blockers (ARBs) are employed as first line therapy in current clinical practice for the management of DN.[18]

Lisinopril is a structural analogue of enalaprilat and is ACE blocker, preventing the conversion of Angiotensin I to a potent vasoconstrictor angiotensin II.[19] Literature data has showed that Lisinopril has a good antiproteinuric effects in DN patients. However, there was a wide variability in individual response when Lisinopril was used for protein lowering effects.[20] It has been observed that association of ACE gene polymorphism with DN is an ethnicity-based problem[21] and we are going to determine possible association between ACE gene polymorphism and DN in Khyber Pakhtunkhwa ethnic population of Pakistan as well to find out the response of Lisinopril in DN patients based on genetics in the form of decreasing micro-albuminuria.

2. Material and Methods

2.1. Study population

The study was approved by Khyber Medical University Peshawar Pakistan – Ethics Board, vide letter no. DIR/KMU-EB/AA/000429. A total number of 242 individuals (DN, n = 121; DM, n = 60; and control, n = 61) were enrolled in the study. Sampling was done from Khyber Teaching Hospital Peshawar Pakistan and further experimental work was done in Institute of Basic Medical Sciences (IBMS), Khyber Medical University Peshawar Pakistan. Patients aged 50 to 70 years, with DM of more than 5 years were included in the study. Patients enrolled were already diagnosed with DN along with micro-albuminuria. Patients having past history of kidney disease were excluded from the study. Demographic and clinical data was taken from all patients on detailed Performa containing information like name, age, gender, ethnicity, duration of disease, contact number and clinical record of the patients, along with the consent form. The demographic and clinical data were obtained at the time of blood sampling. 3 to 5 mL blood was taken by venipuncture and transferred to ethylenediaminetetraacetic acid (EDTA) tubes and kept at -20 ^{o}C for further analysis. The physiological and clinical variables measured were including weight, height, body mass index (BMI) [weight in Kg / Height in m^2], systolic blood pressure (SBP), diastolic blood pressure (DBP), hemoglobin A1c (HBA1c) by fast ion exchange resin method, and cholesterol, serum urea, and creatinine by enzymatic colorimetric method. Micro-albuminuria of all subjects was measured by Immunoterbudometric assay after 24 hours urine collection. The patients included were taking Lisinopril 20 mg twice a day (BD) for two months. After follow-up period of two months, all patients were subjected to the measurement of micro-albuminuria with same method and same laboratory.

2.2. Determination of ACE I/D Polymorphism

After 12 hours or overnight fasting 3-5 mL blood was taken from patients in EDTA tubes. The genomic DNA was extracted from peripheral blood lymphocytes using salting out technique.[22] For identification of ACE I/D polymorphism, polymerase chain reaction (PCR) was performed of all samples. Extracted DNA amplification was carried out in 20 uL volume. The reaction mixture contained master mix 10 uL, deionized water 8.9 uL, primer forward 0.3 uL, primer reverse 0.3 uL and DNA template 0.5 uL. The forward primer was 5'-CTGGAGAGCCACTCCCATCCTTTCT-3' and reverse primer was 5'- GACGTG GCCATCACATTCGTCAGAT-3' were used. PCR cycling conditions were initial denaturation at 94 ^{o}C for 5 minutes 1 cycle, followed by 35 cycles at 94 ^{o}C for 1 minute (melting). Conditions for annealing were 58 ^{o}C for 45 seconds, extension at 72 ^{o}C for 1 minute and final extension at 72 ^{o}C for 8 minutes 1 cycle. The amplified products were analyzed on 2% agarose gel containing 5 uL ethidium bromide. Gel was visualized under UV Transilluminator. Three different types of genotypes bands were identified on gel. Bands at 190bp were DD homozygous, at 490bp II homozygous and at 190bp and 490bp both were D/I heterozygous.

2.3. Sequencing

Twelve random samples were taken for sequencing. Finch TV software was used for analysis. There were no peaks in all chromatograms of 'N' basecaller, so no SNP found in any sample.

2.4. Statistical analysis

Statistical Package for Social Sciences (SPSS) version 20.0 and Microsoft Excel 2013 were used to analyze the means and standard deviations (SD) of DN, DM and control cases.

Demographics data of all groups as well their percentages and genotype frequencies are measured. For continuous variables, normal distribution was verified by the Shapiro-Wilk normality test and the one-sample Kolgomorov-Smirnov test. For normally distributed variables, hypotheses regarding differences among the groups were compared by means of the Welch two sample t-test or by means of the analysis of variance (ANOVA) under general linear model. For non-normally distributed variables, hypotheses regarding differences among the groups were compared by means of the Wilcoxon rank sum test with continuity correction or by means of the Kruskal-Wallis rank sum test.

Yate 's Chi-square test was used for the determination of possibility of ACE gene I/D polymorphism as a risk factor for DN. Risks was reported as odds ratios (OR) along with their 95% confidence interval (CI). Tests in which the p value was smaller than the type I error rate $\alpha = 0.05$ were declared significant.

3. Results

The study included a total of 242 individuals (121 subjects of DN, 60 of DM and 61 healthy subjects). The subjects in DN were 55% female and 45% male while in DM group 40% and 60% were females and males, respectively. In control group, 49% subjects were females and 51% were males. The p-value between male and female in DN, DM and control was 0.0020. The mean, SD, range and p-value of age, weight, height, and BMI of DN, DM and control are shown in Table 1.

DN, Diabetic nephropathy; DM, Diabetes mellitus; BMI, Body Mass Index.

The biochemical analysis for DN is summarized inTable 2. The mean, SD and range of initial and final micro-albuminuria, serum creatinine, urea, HBA1c, SBP, DBP and Cholesterol in DN subjects were 204.85mg/24hr ± 64.72, 220, 161.55mg/24hr ± 58.54, 217, 6.38mg/dl ± 3.73, 21.94,145.48mg/dl ± 76.38, 361, 8.99% ± 2.88,11.5, 142.27mmHg ± 20.07, 130,81.03mmHg ± 14.25, 80, 180.07mg/dl ± 90.89, 660 respectively. The mean, SD, and range of initial micro-albuminuria of DD, II and DI genotype were 214.29 ± 55.00, 165, 206.88 ± 69.63, 204 and 196.47 ± 71.74, 240 respectively. The average, SD, and range of final micro-albuminuria of DD, II and DI genotype were 147.27 ± 55.51, 210, 181.29 ± 58.14, 171 and 163.43 ± 60.55,209 respectively.

HBA1c, hemoglobin A1c; SBP, systolic blood pressure; DBP, diastolic blood pressure; DD, deletion homozygous; II, insertion homozygous; DI, deletion insertion heterozygous.

Using Lisinopril 10 mg BD for two months, the percent decrease in micro-albuminuria was 31.27% 12.37% and 16.81% in DD, II and DI genotype groups, respectively with p-value 0.22 (Table 2). Microalbuminuria can be reversed, and further development of DN reduced. Worldwide screening and timely therapeutic intervention for microalbuminuria is now becoming standard of care. In DN patients the DD, II and DI genotypes were 47 (38.84%), 17 (14.04%) and 57 (47.10%) respectively. In DM group the DD, II and DI were 4 (6.66%), 25 (41.66%) and 31 (51.66%) respectively while in control the DD, II and DI genotype were 38 (62.29%), 1 (1.63%) and 22 (36.06%) respectively as summarized in Table 3 and shown inFigure 1.

DN, Diabetic nephropathy; DM, Diabetes mellitus; DD, deletion homozygous; II, insertion homozygous; DI, deletion insertion heterozygous.

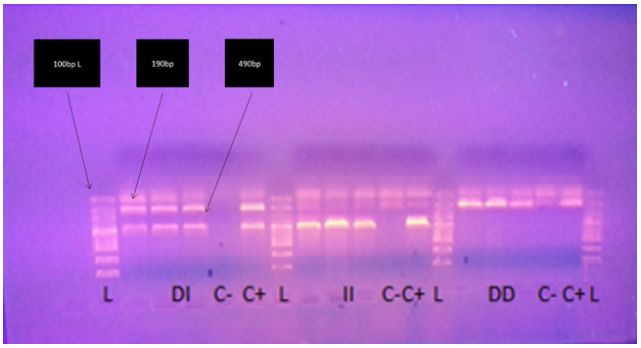

Fig. 1: Gel electrophoresis which shows the Amplification of ACE I/D polymorphism. L indicate ladder of 100bp, C+: Positive control, C-: Negative control, 190bp: DD homozygous, 490bp II homozygous and 190bp and 490bp is D/I heterozygous.

The DD genotype (in comparison to II genotype) was found to have significant association with DN ($p < 0.05$). Table 4 and Figure 2 presented the comparison of genotypes DD and II in DN and DM.

DN, Diabetic nephropathy; DM, Diabetes mellitus; DD, deletion homozygous; II, insertion homozygous; χ^2, chi square; OR, odd ratio; 95% CI, 95% confidence interval.

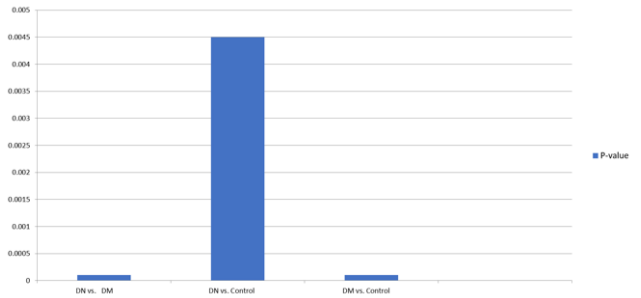

Fig. 2: P-value between DN vs. DM, DN vs. Control and DM vs. Control.

These findings suggest that DD genotype is significantly associated with DN but not a risk factor for development of disease.

Table 1: Various characteristics of diabetic nephropathy patients, diabetes mellitus patients and healthy control

	DN n=121	**DM** n=60	**Control** n=61	**P-value**
Gender (M/F)	54/67	36/24	31/30	0.0020
Age				
Mean ± SD	58.00±7.23	55.42±5.18	56.95±7.25	0.0557
Range	20	20	20	
Weight				
Mean ± SD	69.62±8.23	77.31±14.72	70.47±10.97	<0.0001
Range	37	56	40	
Height				
Mean ± SD	169.46±7.38	166.55±8.81	165.21±8.73	0.0021
Range	28	38	28	
BMI				
Mean ± SD	24.35±3.19	28.09±5.28	25.08±3.72	<0.001
Range	15.34	21.27	19.8	

Table 2: Biochemical analysis of diabetic nephropathy

Investigation				**Values**
July 2017 micro-albuminuria (mg/24h) Mean ± SD				204.85±64.72
Range				220
Septumber 2017micro-albuminuria (mg/24h) Mean ± SD				161.55±58.54
Range				217
Serum creatinine (mg/dl) Mean ± SD				6.38±3.73
Range				21.94
Urea (mg/dl) Mean ± SD				145.48±76.38
Range				361
HBA1c (%) Mean ± SD				8.99±2.88
Range				11.5
SBP (mmHg) Mean ± SD				142.27±20.07
Range				130
DBP (mmHg) Mean ± SD				81.03±14.25
Range				80
Cholesterol (mg/dl) Mean ± SD				180.07±90.89
Range				660
Lisinopril response in DD, II and DI genotype patients				
Micro-albuminuria	**DD**	**II**	**DI**	**P-value**
July 2017 value Mean ± SD Range	214.29±55.00 165	206.88±69.63 204	196.47±71.74 240	0.22
September 2017 value Mean ± SD	147.27±55.51	181.29±58.14	163.43±60.55	
Range	210	171	209	
% decrease	31.27	12.37	16.81	

Table 3: Genotypes distribution among diabetic nephropathy, diabetes mellitus and control groups

Groups	**DD** – n (%)	**II** – n (%)	**DI** – n (%)
DN (N=121)	47 (38.84)	17 (14.04)	57 (47.10%)
DM (N= 60)	4 (6.66)	25 (41.66)	31 (51.66%)
Control (N=61)	38 (62.29)	1 (1.63)	22 (36.06)

Table 4: Comparison of genotype (DD and II) inDiabetic nephropathy, Diabetes mellitus and control groups

Study Groups	Yate 's χ^2 value	Degree of freedom	OR	95% CI	P-value
DN vs. DM	26.31	1	17.28	5.313-49.58	<0.0001
DN vs. Control	8.085	1	0.07276	0.00677-04788	<0.0045
DM vs. Control	45.79	1	0.004211	0.0004048-0.03799	<0.0001

4. Discussion

The diagnosed cases of DM worldwide were 30 million in 1985 which were increased to 135 million up to 1995 and reached to 366 million in 2011. It is expected that it will be 642 million by the year 2040.[23] Untreated cases of DM2 may lead to complications like retinopathy, neuropathy, nephropathy and cardiovascular disorders.[5] DN prevalence is 30–40% in DM2 patients.[24] DN can be identified in early stages by insistent micro-albuminuria, where the excretion rate of albumin could be in the range of 20-200 μg/min or 30-300mg/24 hours.[25] In the present study, using a relatively large sample of patients with DN, DM, and control, it was found that DD genotype has a strong association with DN but not a risk factor for the development of disease. Moreover, the percent decrease of micro-albuminuria with the intake of Lisinopril (10mg two times a day for two months) in DD, II and DI genotype of patients with DN were 31.27%, 12.37% and 16.81% respectively (p-value > 0.05). However, remains controversial association of I/D polymorphism of ACE gene with DN has been reported by studies.[26] A study on Chinese population showed that ACE I/D polymorphism is associated with DN. The ID and DD genotype patients were more prone to DN as compared to genotype II. The p value was 0.001 with 95% CI and ORs of ID and DD were 1.72 (1.10-2.68) and 1.73 (1.27-2.36) respectively.[27] A study in Pakistan on Punjabi ethnic population showed that the frequency of D allele of ACE gene was 40.43% in control and 49% in DN patients, where the DD genotype was found to be 29.7% in DN patients and 18.1% in control. Statistically the difference is not significant (p > 0.05) in comparison to II and DI genotypes. The ACE I/D polymorphism variation was (OR=0.51 and p=0.1). Such findings clearly showed that there is no association of ACE I/D polymorphism with DN.[28] Another study in performed in Pakistan indicated ACE genotype in 168 DN patients as DD (n = 52; 30.95%), II (n = 18; 10.71%) and DI (n = 98; 58.33%), while in DM patients (n = 296) it was reported as DD (n = 25; 8.45%), II (n = 123; 41.55%) and DI (n = 148; 50%). The control group showed DD, (n = 60; 40%) II (n = 42; 28%) and DI (n = 48; 32%) in 150 healthy individuals. The distribution of D allele was (60%) in DN patients and was significantly high as compared to DM patients (33.45%). The comparison of II and DD in DN vs DM group showed that p = 0.0167 and OR 0.3799 with 95%CI 0.2119-0.6811. The II and DD distribution in DN vs control group was p = 0.3608, OR 2.4093 and 95%CI 1.346-4.3125, while between DM and control the distribution of II and DD was p =.00000239, OR 3.6585 and 95% CI 2.1436-6.2441. These finding showed that there is a significant association of DD genotype with DN.[29] To find out possible association between ACE I/D polymorphism with DN, total 242 individuals were included, DN patients (n = 121), DM patients (n = 60) and control (n = 61). In DN patients the DD, II and DI genotypes were 47 (38.84%), 17 (14.04%) and 57 (47.10%) respectively. In DM group the DD, II and DI were (n = 4; 6.66%), (n = 25; 41.66%) and (n = 31; 51.66%) respectively while in control the DD, II and DI genotype were (n = 38; 62.29%), (n = 1; 1.63%) and (n = 22; 36.06%) respectively. Yate 's Chi-square test showed a significant association between ACE I/D and DN (p < 0.05, OR 17.28), suggesting that ACE I/D polymorphism is not a risk factor for the onset of DN. Mogensen and colleagues treated patients with hypertension, microalbuminuria and DM2 with candesartan (16 mg) and Lisinopril (20 mg) once daily, and they noted a 30% decrease in microalbuminuria for candesartan (p < 0.001) and 46% for Lisinopril (p < 0.001).[30] In present study the percent decrease of micro-albuminuria after two months intake of ACE inhibitor (Lisinopril) in DD, II and DI genotypes of patients with DN were 31.27%, 12.37% and 16.81% respectively. It showed that Lisinopril has a good renoprotective effect especially in those individuals who are DD genotype and with higher amount of ACE. Inhibition of ACE with Lisinopril can significantly decrease microalbuminuria.

5. Conclusion

ACE I/D gene polymorphism is significantly associated with the development of DN. The results showed that ACE I/D gene polymorphism is associated with DN, but the OR shows that ACE I/D gene polymorphism is not only the risk factor for the development of DN in which DD genotype is producing more amount of ACE in comparison of II genotype (which is producing less amount of ACE). Various studies have reported controversial results on the association of ACE I/D gene polymorphism with DN, where all studies concluded the reason of ethnicity for the variable results. In our study the results showed that individuals with DD genotype are more prone to DN, while producing higher concentration of ACE. We concluded that the ACE inhibitor Lisinopril has an anti-microalbuminuric effects in patients with DN by inhibiting the activity of ACE enzyme. This

effect appears to be dose related and significantly related to the genotype, as Lisinopril showed a good response in decreasing micro-albuminuria in individuals with DD genotype. Plasma level of ACE is required to measure. Larger sample size is recurred. Multi-center population-based studies are required for validation of findings.

6. Source of Funding

None.

7. Conflict of Interest

None.

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