Original Article

Association Of Genetic Variants Rs1888747 And Rs10868025 Of FRDM3 Gene With Diabetic Neuropathy In Pakistani Patients

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DOI: 10.47750/pnr.2023.14.03.341

Abstract

Background: Diabetic kidney disease (DKD) may cause renal failure, in up to 26% of the patients who are undergoing dialysis for kidney disease. The gene transmission process is not completely understood but it is supposed to be polygenic mechanism and expresses itself in preferable environment suggesting gene-environment interaction. Genome wide-association (GWAS) studies have provided mixed results about gene expression.

Methodology: A case-control study methodology was be implemented in this study. 1050 patients of diabetes mellitus type 2 (DMT2) were selected from Pakistan Institute of Medical Sciences PIMS, department of medicine. The cases were divided into two groups of early and advanced DKD (albuminuria 30-299mg/24h and ≥300mg/24h respectively). There were 370 controls, 352 cases with early DKD and 328 cases with advanced DKD. DNA was isolated from peripheral blood and FRMD3 expression in kidney tissue samples was studies through kidney biopsy samples.

Results: Among all types of SNPs one was significantly associated with the particular disease and that was rs1888747, rs10868025 did not show any association on statistical analysis. The associations were observed in recessive models. To perform regression, the model wars adjusted for diabetes typeII duration, gender of the patient, BP readings and lipid file, the association still persisted. None of the other NSPs were found associated with this disease

Conclusion: The FRMD3 protein expression negatively correlates with FRMD3 genes, so it is good candidate gene for the development of albuminuria. Further exploration is suggested to determine the exact mechanisms for correlation.

INTRODUCTION

Diabetes mellitus is characterized by increased blood sugar levels consistently. The high amount of glucose in body affects different organs like kidney causing diabetic kidney disease. Diabetic kidney disease (DKD) may cause renal failure, in up to 26% of the patients who are undergoing dialysis for kidney disease.(1–5) Diabetic kidney disease also

affects the cardiovascular tissues causing mortality and morbidity after cardiac surgery.(6,7) The development of diabetic kidney disease has genetic basis and there has been a lot of research on it recently.(8,9) The gene transmission process is not completely understood but it is supposed to be polygenic mechanism and expresses itself in preferable environment suggesting gene-environment interaction.(8–13) Genome wide-association (GWAS) studies have provided mixed results about gene expression. GWAS has identified 13 SNPs associated with DKD in two different populations having diabetes type I.(14)

The strongest association is of genes on FRMD3 locus. Other gene loci were also studied for associations. The populations affected with type I or type II diabetes mellitus may share a common gene loci related to DKD. (15–17)Our study will observe whether the SNPs that are associated with type I are also associated with type II diabetes or not. It will also evaluate the genes expressions from kidney biopsies and find the associations of SNPs with early and advanced stages of DKD.

METHODOLOGY

A case-control study methodology was be implemented in this study. 1050 patients of diabetes mellitus type 2 (DMT2) were selected from Pakistan Institute of Medical Sciences PIMS, department of medicine. The cases were defined according to WHO definition of Diabetes mellitus; (18)patients with diabetes diagnosed at or above 35 years of age, no insulin administration for one year after diagnosis and no ketoacidosis. 1050 patients were further divide in to cases and controls. The controls were those patients who had no DKD and whose albumin was <30mg/24h. The cases were divided into two groups of early and advanced DKD (albuminuria 30-299mg/24h and ≥300mg/24h respectively). There were 370 controls, 352 cases with early DKD and 328 cases with advanced DKD. The study protocol was presented to ethical committee of PIMS and executed after approval. Consent forms were signed by all the patients.

The patients were evaluated physically and information was obtained about patient's age at diagnosis, drug treatment, smoking, history of other medical conditions and there treatment. The body mass index for each patient was calculated after measuring weight and height carefully. For cases with advanced DKD, the weight for dialysis dependent patients was measured by calculating mean of weights after dialysis in three consecutive sessions. Hypertension (BP≥140/90mmHg) records were obtained along with medication for BP. The blood samples were collected in fasting. Glucose oxidase method was used to determine fasting plasma glucose levels. Serum creatinine levels were measured through laboratory tests. Cholesterol and triglycerides were measured by enzyme methods. Immunoturbidimetry with intra and inter-assays was performed for urine analysis (urinary albumin excretion UAE).(19–22)

DNA isolation and genotyping

DNA was extracted by salt-out procedure from peripheral leukocytes. Our gene of interest was rs1888747 and rs10868025 both located on the same locus on 9q. Human Custom TaqMan Genotyping assays were used. Real time polymerase chain reaction RT-PCR was done in 96-well plates with primers and probes by using 5 microliters reaction solution and 2 nan-grams of DNA genome. The plates were placed on RT-PCR cycler on 95 degree Celsius and heated for 10 minutes. This was followed by 45 cycles on 95 degree for 15-20 seconds and then on 60 degree for one minute. The success rate was 95% with 0.01% calculated error. FRMD3 expression in kidney tissue sample. The DNA samples of patients with advanced DKD obtaining treatment for nephropathy during last one year were taken. Complete history of the patient was considered before DNA collection that included record of any previous diseases, record of nephrectomy, time since the diagnosis of diabetes mellitus and records of hypertension and smoking. mRNA expression using western blot (WB) and protein expression using immunohistochemistry (IHC) was performed for DNA samples obtained from kidney tissues. The kidney tissues was obtained after consent of ethical committee of hospital and written informed consent of the patient. Out of 90 samples collected, 45 samples were chosen for DNA genotyping others could not be used because the tissues were not enough.

Kidney biopsies were contained in phenol-isothiocyanate.(23) The RNA was extracted and centrifuged (12,000xg) with chloroform and isopropanol respectively. The precipitated RNA was then washed and suspended with ethanol (75%) and 10 – 50 microliter of diethylpyrocarbonate. The isolated RNA was then assessed on NANODROP

2000spectophotometer.(24) The mean and standard deviation was 2.58±1.47µg/250 for the concentrated RNA obtained from kidney.

RT-PCR reverse transcription was done in two parts. The RNA was transcribed into cDNA and cDNA was amplified through reverse PCR transcription. The kit used was Super Script Vilo Master Mix Kit and the experiment was done in 7500 real time polymerase chain reaction system. 10µl of SYBER Green solution was used in PCR reaction with 1µl of gene specific primer (FRMD3), 7µl of water and 1µl cDNA. Three thermo-cycles were performed, first on 95 degree for ten minutes, then fifty cycles on 95 degree for 15 seconds each and last cycle on 95 degrees for one and half minutes. ΔΔCq method was used to quantify FRDM3(25,26) cDNA and it was expressed to cyclophilin A in 90 kidney tissue samples, 74 of which were carrying G allele of rs1888747 SNP, and 16 with CC genotype. cDNA sample was diluted and amplification was done separately for FRDM3 and cyclophilin A. Equal amplification efficiencies were applied in all experiments (95-105%). ΔΔCq method gives values for changes in gene expression with each fold. (27)

FRMD3 gene distribution was determined in forty seven samples due to technical limitations and inadequate sample. 39 of these samples carried allele for rs1888747SNP and 7 contained rs10868025.

To find the association between cases and controls first the cases and controls were kept into two different groups and then cases divided into further two groups with early and advanced diabetic kidney disease data were analyzed for best fit all frequencies of alleles of SNPs were calculated by counting genes and the distributions were verified using chi-square test. The distribution of genotype frequencies and alleles were compared using chi-square test. Mean and standard deviation values were used for continuous variables and medians were taken for the variables with skewed distributions. Categorical data were expressed in numbers of cases and percentage of individuals affected and ANOVA or one way analysis of variance. Chi-square test or student t-test were performed to evaluate the difference is clinical and laboratory representation of FRMD3 gene expression. Association magnitude was determined using odds ratio with 95% confidence interval (CI). Multinomial logistic regression analysis was used to determine the possibility of association of SNPs directly to kidney disease, co-variables were adjusted. Pearson chi-square value of correlation was used do valuate the correlation between variables. Multiple layered linear regression models were applied to FRMD3 gene as dependent variable and age and sex as independent variables. Result values less than 0.05 were considered significant. All analysis were performed as on SPSS

RESULTS

We evaluated 1050 patients of diabetes type two out of which 352 had diabetic kidney disease (DKD) of early stage and 328 had advanced diabetic kidney disease (DKD) and they were called cases. Other 370 patients were controls. Patients who had diabetic kidney disease (CKD) had bad profile in terms of blood pressure records and a bad lipid profile when compared to control group. Among all types of SNPs one was significantly associated with the particular disease and that was rs1888747, rs10868025 did not show any association on statistical analysis. The associations were observed in recessive models. To perform regression, the model wars adjusted for diabetes typeII duration, gender of the patient, BP readings and lipid file, the association still persisted. None of the other NSPs were found associated with this disease.

FRMD3 expression study

Since only rs1888747 was associated with diabetic kidney disease in this sample of subjects with diabetes type 2 and this SNP is located near FRMD gene we concluded that FRMD gene and protein expression in human kidney tissue is important to determine the prevalence of DKD. The mean age of 90 subjects for rs1888747 NSPs was 57.7±14.2. 57.8% were male, 50.4% had arterial HTN 22.8% were smokers and 18.9% had bad lipid profile.

Table 1 Genotype distribution of rs1888747

	Renal status		

Genotype	Normal albuminuria N=370	Micro albuminuria N=352	Macro albuminuria N=328	P*	P**
CC	40 (10.18)	27 (7.7)	23 (7.01)	0.04	0.05
GC	152 (41.08)	148 (42.04)	146 (44.51)		
GG	178 (48.10)	177 (50.28)	159 (47.56)		
C allele	0.31	0.28	0.27		
The distribution is expressed as numbers and percentages P* is obtained by all comparing all three groups P** is obtained by comparing micro and macro-albuminuria					

Table 2 Genotype distribution in cases and controls

Polymorphism	Controls (n = 370)	Cases (n = 680)	P	Dominant: OR (95 % CI)/P	Recessive: OR (95 % CI)/P	Additive: OR (95 % CI)/P
CC	10.18 (40)	50 (7.4)	0.037	0.81 (0.71–1.05)/0.032	0.59 (0.40–0.93)/0.028	0.59 (0.37–0.89)/0.029
CG	41.08 (152)	294 (43.2)				
GG	48.10 (178)	336 (49.41)				
The distribution is expressed as frequencies and percentages						

Since rs1888747 was the only SNP associated significantly with DKD (CC/CG/GG = cases vs. controls = 7.4%/50 vs 10.18%/40 P = 0.037). Minor allele frequencies were 0.31 and 0.55 in controls and cases (microalbuminuric + macroalbuminuric/ESRD), respectively (P = 0.06). The strongest association was observed assuming recessive (CC vs. CG/GG, OR: 0.59, 95 % CI 0.40–0.93; P = 0.028) and additive (CC vs. GG, OR: 0.59, 95 % CI 0.37–0.89; P = 0.029) models. This association persisted after controlling for T2DM duration, gender, systolic BP, and triglycerides profile. The other SNPs are not associated with DKD including rs10868025. No other allele was precipitated or showed positive results so it is assumed that they were not expressed in DKD patients.

DISCUSSION

In our study, it was found that rs1888747 is positively associated with DKD in type 2 diabetes mellitus; it is associated with type1 diabetes as stated in previous studies.(15) The DKD patients were further divided into two groups to explore the reasons for development of DKD. The CC genotype is dominant in microaluminuria and shows protective behavior. GWAS has explored many unknown genes and alleles associated with DKD but these studies were not replicated for different populations. (13)(28,29)The gaps in the studies have led to lesser evidences on the causation of the condition and association of the genes. rs1888747 SNP is associated with development of DKD but the populations studies in different ethnic regions have produced confusing results. (30–34)

GWAS has been studying genes on different locations, there have been advances in genetic engineering and various genotypes are studies over the years but the exact pathogenesis of DKD remains elusive. Gene-environment interactions, phenotypes, different characteristics of populations. This study also aimed at evaluating risk related to rs1888747 alleles by determining the influence on FRMD3 gene expression. FRMD3 is located on 9q21.32. The FRMD3 protein expression negatively correlates with FRMD3 genes, so it is good candidate gene for the development of albuminuria. Further exploration is suggested to determine the exact mechanisms for correlation.

References:

1. Foley RN, Collins AJ. End-stage renal disease in the United States: an update from the United States Renal Data System. J Am Soc Nephrol [Internet]. 2007;18. Available from: https://doi.org/10.1681/ASN.2007020220
2. Bloomgarden ZT. Diabetic nephropathy. Diabetes Care [Internet]. 2008;31. Available from: https://doi.org/10.2337/dc08-zb04
3. Prevention., C.f.D.C.a., National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States. 2011, Atlanta, GA: US Department of Health and Human Services.
4. Tapp RJ. Albuminuria is evident in the early stages of diabetes onset: results from the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). Am J Kidney Dis [Internet]. 2004;44. Available from: https://doi.org/10.1016/S0272-6386(04)01079-0
5. Lim A. Diabetic nephropathy—complications and treatment. Int J Nephrol Renov Dis [Internet]. 2014;7. Available from: https://doi.org/10.2147/IJNRD.S40172
6. Bruno RM, Gross JL. Prognostic factors in Brazilian diabetic patients starting dialysis: a 3.6-year follow-up study. J Diabetes Complicat [Internet]. 2000;14. Available from: https://doi.org/10.1016/S1056-8727(00)00118-5
7. Valmadrid CT. The risk of cardiovascular disease mortality associated with microalbuminuria and gross proteinuria in persons with older-onset diabetes mellitus. Arch Intern Med [Internet]. 2000;160. Available from: https://doi.org/10.1001/archinte.160.8.1093
8. Krolewski AS. A genome-wide linkage scan for genes controlling variation in urinary albumin excretion in type II diabetes. Kidney Int [Internet]. 2006;69. Available from: https://doi.org/10.1038/sj.ki.5000023
9. Vardarli I. Gene for susceptibility to diabetic nephropathy in type 2 diabetes maps to 18q22.3-23. Kidney Int [Internet]. 2002;62. Available from: https://doi.org/10.1046/j.1523-1755.2002.00663.x
10. Leitao CB. The role of K121Q ENPP1 polymorphism in diabetes mellitus and its complications. Braz J Med Biol Res [Internet]. 2008;41. Available from: https://doi.org/10.1590/S0100-879X2006005000202
11. Canani LH. The fatty acid-binding protein-2 A54T polymorphism is associated with renal disease in patients with type 2 diabetes. Diabetes [Internet]. 2005;54. Available from: https://doi.org/10.2337/diabetes.54.11.3326
12. Murea M. Genome-wide association scan for survival on dialysis in African-Americans with type 2 diabetes. Am J Nephrol [Internet]. 2011;33. Available from: https://doi.org/10.1159/000327985
13. Thameem F. A genome-wide search for linkage of estimated glomerular filtration rate (eGFR) in the Family Investigation of Nephropathy and Diabetes (FIND). PLoS One [Internet]. 2013;8. Available from: https://doi.org/10.1371/journal.pone.0081888
14. Mueller PW. Genetics of Kidneys in Diabetes (GoKinD) study: a genetics collection available for identifying genetic susceptibility factors for diabetic nephropathy in type 1 diabetes. J Am Soc Nephrol [Internet]. 2006;17. Available from: https://doi.org/10.1681/ASN.2005080822
15. Pezzolesi MG. Genome-wide association scan for diabetic nephropathy susceptibility genes in type 1 diabetes. Diabetes [Internet]. 2009;58. Available from: https://doi.org/10.2337/db08-1514
16. Nathan DM. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. N Engl J Med [Internet]. 2005;353. Available from: https://doi.org/10.1056/NEJMoa052187
17. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med [Internet]. 1993;329. Available from: https://doi.org/10.1056/NEJM199309303291401
18. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care. 2003; 26(Suppl 1): S5–20.
19. Canani LH, Gerchman F, Gross JL. Increased familial history of arterial hypertension, coronary heart disease, and renal disease in Brazilian type 2 diabetic patients with diabetic nephropathy. Diabetes Care [Internet]. 1998;21. Available from: https://doi.org/10.2337/diacare.21.9.1545
20. Camargo JL, Felisberto M, Gross JL. Effect of pre-analytical variables on glycohemoglobin measurements in routine clinical care. Clin Biochem [Internet]. 2004;37. Available from: https://doi.org/10.1016/j.clinbiochem.2004.03.016
21. Junge W. Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffe method. Clin Chim Acta [Internet]. 2004;344. Available from: https://doi.org/10.1016/j.cccn.2004.02.007
22. Zelmanovitz T. The receiver operating characteristics curve in the evaluation of a random urine specimen as a screening test for diabetic nephropathy. Diabetes Care [Internet]. 1997;20. Available from: https://doi.org/10.2337/diacare.20.4.516
23. Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. Am J Clin Pathol. 1981;75.
24. Bustin SA. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem [Internet]. 2009;55. Available from: https://doi.org/10.1373/clinchem.2008.112797
25. Higuchi R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnol (N Y) [Internet]. 1993;11. Available from: https://doi.org/10.1038/nbt0993-1026
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods [Internet]. 2001;25. Available from: https://doi.org/10.1006/meth.2001.1262
27. Brondani LA. The UCP1-3826A/G polymorphism is associated with diabetic retinopathy and increased UCP1 and MnSOD2 gene expression in human retina. Invest Ophthalmol Vis Sci [Internet]. 2012;53. Available from: https://doi.org/10.1167/iovs.12-10660
28. Palmer ND. Evaluation of candidate nephropathy susceptibility genes in a genome-wide association study of African American diabetic kidney disease. PLoS One [Internet]. 2014;9. Available from: https://doi.org/10.1371/journal.pone.0088273
29. McKnight AJ, McKay GJ, Maxwell AP. Genetic and epigenetic risk factors for diabetic kidney disease. Adv Chronic Kidney Dis [Internet]. 2014;21. Available from: https://doi.org/10.1053/j.ackd.2014.03.010

30. Mooyaart AL. Genetic associations in diabetic nephropathy: a meta-analysis. Diabetologia [Internet]. 2011;54. Available from: https://doi.org/10.1007/s00125-010-1996-1
31. Freedman BI. Differential effects of MYH9 and APOL1 risk variants on FRMD3 Association with Diabetic ESRD in African Americans. PLoS Genet [Internet]. 2011;7. Available from: https://doi.org/10.1371/journal.pgen.1002150
32. Pezzolesi MG. Family-based association analysis confirms the role of the chromosome 9q21.32 locus in the susceptibility of diabetic nephropathy. PLoS One [Internet]. 2013;8. Available from: https://doi.org/10.1371/journal.pone.0060301
33. Maeda S. Replication study for the association between four Loci identified by a genome-wide association study on European American subjects with type 1 diabetes and susceptibility to diabetic nephropathy in Japanese subjects with type 2 diabetes. Diabetes [Internet]. 2010;59. Available from: https://doi.org/10.2337/db10-0067
34. Williams WW. Association testing of previously reported variants in a large case-control meta-analysis of diabetic nephropathy. Diabetes [Internet]. 2012;61. Available from: https://doi.org/10.2337/db11-0751.