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Vitamin D receptor *Fok*I polymorphism in a Pakistani population with type 2 diabetes mellitus

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Abstract: Present research was undertaken with the aim to assess the association of VDR gene *Fok*I polymorphism with T2DM in local population. The study comprised of 100 T2DM patients (DG) and 50 normal individuals (CG) groups. Demographic parameters; age, gender, BMI and blood pressure were recorded. Fasting glucose (FG), HbA1c, vitamin D, liver function parameters, renal function parameters and lipid profile were measured. Significantly higher ($P<0.05$) BMI (34.6±11.3 *vs.* 24.9±4.0kg/m^2), sBP (141±16 *vs.* 124±14mm Hg), dBP (81±8 *vs.* 76±7mm Hg), FG (145±5.54 mg/dL *vs.* 80±3.55mg/dL, HbA1c (7.43±0.69 % *vs.* 4.85±0.33%) were evident in DG as compared to CG. Prominent reduction ($P<0.05$) in vitamin D levels (13.69±1.85mg/dL) manifested in case subjects than that of control subjects (22.36±2.34mg/dL) as a negative correlation existed between HbA1c and vitamin D. Compared to control participants, substantially different *Fok*I allele distribution was observed in T2DM patients. Current study s also showed no significant link between *Fok*I genotype and the biochemical parameters. Present study endorsed the fact that diabetic patients have hypovitaminosis D and variable VDR polymorphisms. However, confirmational studies are indecisive and warrants further research.

Keywords: Type 2 diabetes, hyperglycemia, hypovitaminosis, VDR gene, polymorphisms.

INTRODUCTION

Hypovitaminosis D is a health enigma influencing about one million worldwide. The risk factors include obesity, sedentary lifestyle, limited sunlight exposure and aging. Convincing evidences suggest that hypovitaminosis D is a probable cause for type 2 diabetes mellitus (T2DM) leading to increased mortality (Galesanu and Mocanu, 2015; Matyjaszek-Matuszek *et al*., 2015; Mauss *et al*., 2015; Riaz *et al*., 2016).

Various aspects of glucose metabolism are positively affected by vitamin D. However, a contributory relation is yet not determined (Bajaj *et al*., 2014; Al-Shoumer and Al-Essa, 2015; Herrmann *et al*., 2015; Mathieu, 2015; Usluogullari *et al*., 2015). From serum 25(OH) vitamin D levels, cardiac complications in T2DM can be anticipated (Heidari *et al*., 2015). An opposite association between vitamin D and metabolism and insulin resistance is reported (Calvo-Romero *et al*., 2015; Miñambres *et al*., 2015; McDonnell *et al*., 2016). Jung *et al*. (2015) observed that vitamin D deficiency correlated with heart rate variability parameters in T2DM. However, Sheth *et al*. (2015) stated that hypovitaminosis D was prevalent in non-diabetic subjects.

Vitamin D receptor (VDR) gene variants, specifically *Apa*I, *Taq*I, *Fok*I and *Bsm*I may be linked with diabetes, impaired physiology of insulin and beta cells of pancreas (Ogunkolade *et al*., 2002; Pittas *et al*., 2007). However, inferences of recent evidences are indecisive. VDR polymorphisms are associated with impaired insulin sensitivity (Filus *et al*., 2008) and insulin secretion (Speer *et al*., 2001; Ogunkolade *et al*., 2002). Contrary to these reports, some researchers denied the existence of any similar associations (Malecki *et al*., 2003; Bid *et al*., 2009; Dilmec *et al*., 2010; Vural and Maltas, 2012). This scenario demands analysis to explore the role of VDR polymorphisms in predisposing T2DM.

Vitamin D gene variations are hazardous modifiers in diabetes progression. However, paucity of a case control study among Pakistani population is the main obstacle to draw solid conclusion. Present research was conducted to assess the association of VDR gene *Fok*I polymorphism with T2DM biomarkers in local population.

MATERIALS AND METHODS

Research design

The case-control study comprised of 100 T2DM patients attending private clinics in Faisalabad, Pakistan along with 50 normal individuals during the period January, 2015 to February, 2015 recruited through a convenient sampling technique. Informed written consent was procured and participants were divided into two groups; diabetic group (DG) and control group (CG). Ethical approval of research protocols were procured from Graduates Studies and Research Board (GSRB), University of Agriculture, Faisalabad, Pakistan and research work was conducted in collaboration with Department of Medical and Dentistry, Southmead Hospital, University of Bristol, Bristol, UK.

Exclusion and inclusion criteria

Inclusion criteria for diabetic case subjects was: HbA1c: ≥

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7%, fasting glucose (FG): ≥ 126mg/dL and body mass index (BMI): 25-40kg/m^2, disease history or use of antidiabetic medicines. Inclusion criteria for healthy control subjects was: HbA1c: ≤ 7.0 %, fasting plasma glucose: ≤ 126mg/dL and body mass index (BMI): 18-25kg/m^2 (WHO, 2014). Exclusion criteria was hepatitis B, C, HIV, pancreatic or renal failure, pregnancy or lactation, psychiatric disorder, uncontrolled hypertension, use of medication affecting lipid, calcium and bone metabolism, endocrinopathies (hypo- or hyperthyroidism, parathyroidism), use of anticonvulsant drugs; or use of vitamin D or calcium supplements and taking any medication.

Sample Collection

Participants were interviewed and examined to collect demographic data such as age, gender, weight and height, and blood pressure. Hypertension in terms of blood pressure (BP) was characterized by ≥140mm Hg (systolic), ≥ 90mm Hg (diastolic), or hypotensive therapy. Fasting blood samples taken in EDTA (ethylene diamine tetra acetic acid) coated vacutainers were processed for further bioassays.

Biochemical Assays

FG, vitamin D, total and direct bilirubin, blood urea nitrogen (BUN), creatinine, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were measured using commercially available kits (Merck, Germany). Glycated hemoglobin (HbA1c) estimation kit by Randox, UK was used. Hypovitaminosis D was defined as 25(OH) Dlevels <16mg/dL. Biochemical analyses were done as per manufacturer's instructions using Dade Behring clinical chemistry system for dimension auto-analyzer, Siemens, USA and Diastat auto-analyzer, Randox, UK.

DNA Extraction and quantification

Blood samples (5mL) were collected in EDTA coated vacutainer tubes. Salting out method for genomic DNA (deoxy nucleic acid) extraction was implied using proteinase K. NanoDrop spectrophotometer was used to quantify and assess purity of DNA as OD260/OD280 (Sambrook *et al*., 2000).

PCR primer and Optimization of amplification condition

PCR primers sequences used were (Dilmec *et al*., 2010): (F: CCCTGGCACTGACTCTGCTC; R: GGAAACACC TTGCTTCTTCTCC). Primers were diluted to 1µg/µL stock and these stocks were further diluted to working concentration of 10pmol/µL. The reactions were set in 0.2mL PCR tubes. The PCR reaction mixture (10µL) contained 5X Colorless GoTaq® Flexi Buffer (Part# M890A): Proprietary formulation supplied at pH 8.5. The buffer contains 20 mmol. Tris HCl; pH7.5; 100mmol. NaCl; 0.1mmol. EDTA; 1mmol. dithiothreitol; 1.0µL of 50% (v/v) glycerol. Other materials used were 1.0mmol. dNTPs, 0.3mL of 50mmol. $MgCl_2$, 5pmol/µL primers for nucleic acid (forward and reverse) section (0.4µL each). Go Taq kit (Promega Madison, Wisconsin USA) was used for about 1.0mL of genomic DNA (100ng/µL). PCR conditions were optimized for annealing temperature (60°C) and Mg^{2+} concentration.

Polymerase chain reaction (PCR)

Amplification of exon 2 was performed according to a standard protocol (PCR thermocycler T100TM, BioRad). Amplification cycles were completed after denaturation for 180 seconds at 95°C. Then again denaturation at same experimental conditions and an annealing process for 30 seconds with annealing temperature optimized for each primer set and an extension step for 30 seconds (72°C). Last extension was done for 240 seconds (72°C).

Enzymatic digestion

Amplified segments were subjected to enzymatic digestion at particular temperature and time duration to evaluate the genotypes. For *Fok*I (rs10735810 T>C), the digestion conditions were 37°C incubation temperature (60 minutes) for 70, 197, 267 bp digestion fragments (New England BioLabs®, R0109S). After PCR, individual fragments were digested by a mixture of 1.0µL of the respective enzyme and 5.0µL NE-buffer give appropriate fragments.

Electrophoresis

Agarose gel (3%) for genomic DNA and 2% for PCR amplified product and digested fragments was prepared by boiling agarose with 1X TBE (Tris-Borate-EDTA) and staining was done by using Medori nucleic acid staining solution (Bulldog Bio, USA). For comparison, 100bp plus huge DNA Ladder marker and for visualization UVITEC system was used.

STATISTICAL ANALYSIS

Results were represented as mean ± SD (standard deviation), % or n (number) unless otherwise indicated. SPSS software (version 17) was used for data interpretation with a value of $P<0.05$ as a significant value by one-way-ANOVA.

RESULTS

Biochemical assays

Clinical and biochemical characteristics are summarized in table l. Sample was homogeneous in terms of age and gender. Considerably elevated BMI, sBP, dBP and poor hyperglycemic state ($P<0.05$) was evident in DG group as compared to CG. Considerably elevated ($P<0.05$) HbA1c value was found in DG. Whereas, CG exhibited well-managed HbA1c. Prominent reduction ($P<0.05$) in vitamin D levels manifested in DG subjects than that of CG subjects. A negative association existed between glycated haemoglobin and vitamin D.

Table 1: Demographic and biochemical characteristics of case and control subjects

Parameters	Diabetic group (n = 100)	Control group (n = 50)	*P* value
Clinical			
Age (years)	47.5 ± 5.0	48.3 ± 5.4	>0.05
Gender (Male/Female)	50/50	25/25	>0.05
BMI (kg/m^2)	34.6 ± 11.3	24.9 ± 4.0	<0.05
sBP (mm Hg)	141 ± 16	124 ± 14	<0.05
dBP (mm Hg)	81 ± 8	76 ± 7	<0.05
Biochemical			
FG (mg/dL)	145 ± 5.54	80 ± 3.55	<0.05
HbA1c (%)	7.43 ± 0.69	4.85 ± 0.33	<0.05
Vitamin D (mg/dL)	13.69 ± 1.85	22.36 ± 2.34	<0.05
Renal Function Tests			
Total B (mg/dL)	1.07 ± 0.42	1.10 ± 0.41	0.53
Direct B (mg/dL)	0.93 ± 0.23	0.94 ± 0.23	0.83
Liver Function Tests			
ALT (mg/dL)	70.98 ± 15.19	72.94 ± 15.14	0.32
AST (mg/dL)	33.77 ± 18.76	36.37 ± 19.07	0.29
ALP (mg/dL)	50.91 ± 10.37	50.93 ± 10.37	0.99
Renal Function Tests			
BUN (mg/dL)	40.53 ± 8.34	14.72 ± 4.34	<0.05
Creatinine (mg/dL)	2.02 ± 0.48	0.50 ± 0.33	<0.05
Uric acid (mg/dL)	6.19 ± 0.97	2.89 ± 0.78	<0.05
Lipid Profile			
Cholesterol (mg/dL)	286.24 ± 28.54	178.03 ± 11.96	<0.05
LDL-C (mg/dL)	170.69 ± 10.15	64.40 ± 12.76	<0.05
HDL-C (mg/dL)	36.24 ± 3.25	62.12 ± 14.33	<0.05
TG (mg/dL)	447.39 ± 154.86	180.49 ± 9.06	<0.05

Data expressed as mean ± SD (standard deviation), % or n (number) unless otherwise indicated, P<0.05: significant.

BMI: body mass index, sBP: systolic blood pressure, dBP: diastolic blood pressure, FG: fasting glucose, HbA1c: glycated hemoglobin, Total B: total bilirubin, Direct B: direct bilirubin, ALT: alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase, BUN: blood urea nitrogen, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglycerides

Table 2: Distribution of genotype, allele frequencies and carriage rate of *Fok*I among patients and controls

Genotype	Group		Total
	CG	DG	
Ff	11	13	24
	11.0%	8.7%	9.6%
Ff	36	91	127
	36.0%	60.7%	50.8%
FF	53	46	99
	53.0%	30.7%	39.6%
Total	100	150	250
	100.0%	100.0%	100.0%

Data expressed as $X^2 = 8.33^S$, $p<0.016$

Total and direct bilirubin measurements were almost analogous in both the groups. As for liver enzymes status, ALT, AST and ALP concentrations were almost similar in both diabetic and non-diabetic controls. None of the LFT (liver function test) parameters were related with vitamin D status.

Although RFT (renal function test) assays had negative correlation with vitamin D in present study. Even then, T2DM sample showed appreciably ($P < 0.05$) higher BUN levels when compared with control sample. Likewise, creatinine and uric acid concentrations were found to be remarkably ($P < 0.05$) elevated in these patients as compared to the control participants. As expected, lipid profile results were remarkably different ($P < 0.05$) among case and control participants. Within the each group, associations of biochemical variables with vitamin D were negligible. Raised cholesterol and triglycerides measurements were observed for DG than that of CG. LDL-C, a well-recognized cardiovascular risk marker was at substantially ($P < 0.05$) upper level (170.69±10.15mg/dL) in DG. While its value in CG was 64.40±12.76mg/dL. Contrary to this finding, HDL-C known as good cholesterol, was significantly ($P < 0.05$) lower in DG as when matched up with those of CG.

Table 3: Probability values for the association of biochemical parameters and *Fok*I genotypes in T2DM sub-groups

Parameters	DG	CG
HbA1c	0.606	0.858
Vitamin-D	0.536	0.472
T. bilirubin	0.047	0.631
D. bilirubin	0.791	0.033
ALT	0.151	0.545
AST	0.566	0.839
ALP	0.394	0.262
BUN	0.507	0.011
Creatinine	0.643	0.183
Uric acid	0.949	0.908
Cholesterol	0.811	0.591
LDL-C	0.214	0.251
HDL-C	0.556	0.730
TG	0.025	0.898

Data expressed as *p*–value HbA1c: glycated hemoglobin, T. bilirubin: total bilirubin, D. bilirubin: direct bilirubin, ALT: alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase, BUN: blood urea nitrogen, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, TG: triglycerides

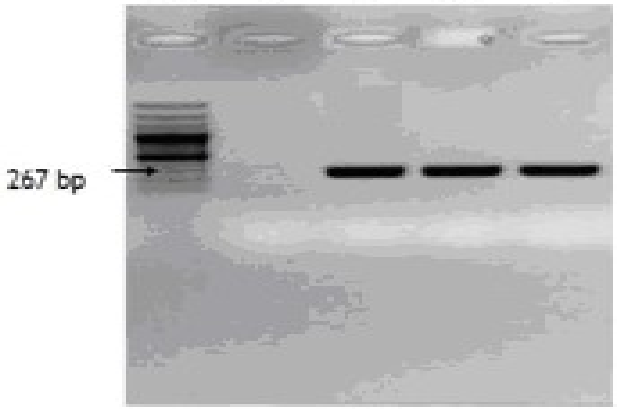

Fig. 1: Electrophoresis of a 2% agarose gel with exon 2 PCR product loaded; 1-negative control; 2, 3 and 4-exon 2 fragment with 267 bp.

FokI polymorphisms

Amplification resulted in a 267 bp fragment (fig. 1). To confirm heterozygosity, enzymatic digestion was done by restriction enzyme, *Fok*I digested fragments are given in fig. 2. In the present study remarkably different *Fok*I allele distribution in T2DM participants group as compared to control participants manifested (table 2). These findings suggest that the *Fok*I polymorphism may contribute to the susceptibility T2DM complications. The distribution of *Fok*I allele genotypes in T2DM complications groups was not statistically different as compared to control group (table 2). Current study has also shown no significant link between *Fok*I genotype and the biochemical parameters (table 3). Thus further studies are required into current scientific domain among local population.

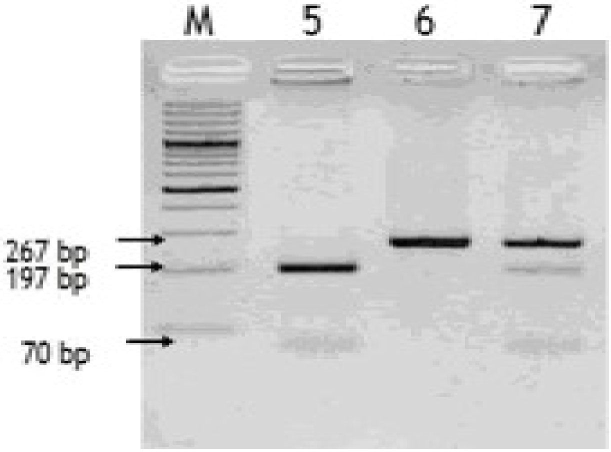

Fig. 2: Electrophoresis of a 3% agarose gel with *Fok*I enzymatic digestion of *VDR* exon 2; 5-homozygous TT genotype (70 and 197 bp); 6-homozygous CC genotype (267 bp); 7-heterozygous CT genotype (70, 197 and 267 bp

DISCUSSION

The levels of vitamin D in normal and diseased conditions is a matter of debate. Vitamin D deficiency is far more pervasive than its overload (Heidari *et al*., 2015; Matyjaszek-Matuszek *et al*., 2015). Although high rates of vitamin D deficiency among Pakistani population are documented (Iqbal and Khan, 2010; Roomi *et al*., 2015; Riaz *et al*., 2016). Literature review reveals neither vitamin D status among individuals residing in Faisalabad.

The major finding of present study was significantly lower vitamin D concentrations in DG as compared to CG. Glycemic control was not satisfactory in case participants. In addition, 25 (OH) D and HbA1c concentrations had an inverse relation (r = -0.56, P<0.05). As expected, other biochemical parameters such as FG, renal, hepatic and function tests and lipid profile showed significant differences among case and control subjects. However, none was found to have any correlation with vitamin D measurements in both the groups. The target organs for vitamin D are liver, kidney, bone and intestine. Abnormalities in RFT, LFT and lipid profile are intermingled among these organs (Mauss *et al*., 2015). Vitamin D deficiency in case and control subjects was analogous Djalali *et al*. (2013).

Hypovitaminosis D in T2DM subjects when compared to healthy subjects is already established by several studies and support our conclusions (Whitfield *et al*., 2005; Bajaj *et al*., 2015). Similar to our findings, Calvo-Romero and Ramiro-Lozano (2015) observed low 25 (OH) D concentrations in diabetics and opposite association between 25 (OH) D and HbA1c. A correlation analysis by Miñambres *et al*. (2015) further supported similar associations in T2DM.

Inverse correlation between glycated hemoglobin and vitamin D deficiency can be justified by the fact that vitamin D intake reduces HbA1c levels in diabetic people (Whitfield *et al*., 2005). Contrary to that, almost analogous 25-OH D concentrations among diabetic and nondiabetic subjects were reported by Usluogullari *et al*. (2015). Likewise, Sheth *et al*., (2015) suggested that though vitamin D deficiency is prevalent in T2DM, its role in hemoglobin glycation could not be established. Disturbance in vitamin D levels is also allied to abnormal glucose metabolism. Though it is unclear whether vitamin affects glucose or vice versa (Al-Shoumer & Al-Essa, 2015).

*Fok*I is considered as another important restriction site of VDR gene polymorphism, located at 5' end of the gene. It may be responsible to alter the structure and function of VDR protein (Filus *et al*., 2008). These polymorphism may be involved in susceptibility of T2DM and its pathogenesis. Previous studies observed higher prevalence of Ff genotype of VDR gene in T2DM patients as compared to control group. *Fok*I restriction site found in exon 2, is involved in transcriptional activity of VDR gene but genetic background of T2DM remains unclear (Wang *et al*., 2010).

*Fok*I polymorphism was found to have no association with various clinical or biochemical parameters, although genetic background of T2DM pathogenesis was not well defined (Bid *et al*., 2009; Dilmec *et al*., 2010). According to Speer *et al.,* (2001) and Malecki *et al.,* (2003), no impact of VDR gene polymorphisms including *Fok*I was found in pathogenesis of T2DM complications.

CONCLUSION

It is concluded from present study that diabetic patients have hypovitaminosis D and variable VDR polymorphisms. However, conformational studies are indecisive and warrants further insight into the complex interplay between T2DM and vitamin D.

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