Original article

Vitamin D receptor gene polymorphism (Fok1 rs2228570) and its complications in Sudanese Patients with Diabetes Mellitus

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Abstract

Background: Diabetes mellitus (DM) is a complicated disease caused by both genetic and non-genetic factors. Vitamin D (*VD*) has a role in a number of functions, including insulin synthesis and secretion regulation that determined by the activation of the vitamin D receptor (*VDR*).

Aim: The goal of this study was to investigate a possible association of Fok1 polymorphism (rs2228570) of the *VDR* gene with DM and its complications in Sudanese Patients.

Methods: A total of 181 diabetic patients and 128 healthy controls were participated in this cross-sectional study. The Fok1 polymorphism (rs2228570) was detected using PCR, followed by RFLP using the Fok1 restriction enzyme.

Result:

The results showed that 64% of the examined diabetic were females with no significant difference (P_value=0.08) in gender between diabetic compared to the healthy controls. Nevertheless, ninety-three percent of the cases were in the age of 40 that had a five-fold greater risk of diabetes compared to controls of the same age group (P_value=0.00, OR= 5.2). Diabetic septic foot (DSF), diabetic neuropathy (DNP), and diabetes retinopathy (DRP) have all been documented among diabetics with prevalence rates of 43%, 24% and 12%, respectively.The genotype analysis of the Fok1 polymorphism (rs2228570) revealed a significant association with diabetic patients compared to the healthy controls (P=0.02), however, there were no significant association for DSF, DN, and DR (p=0.09, 0.21, and 0.10, respectively).The mutant C allele was found in high frequency among DM patients, DSF, DNP, and DRP (80%, 82%, 86%, and 91 %, respectively), however the carriage of the mutant C allele did not differ substantially between diabetic and diabetic complicate patients.

Conclusion: The Fok1 polymorphism (rs2228570) of the *VDR* gene was shown to be related with diabetes in the participants of this study. The high frequency of the mutant C allele in diabetic patients, DSF, DNP and DRP patients could be a predictor of disease onset and consequences in over age Sudanese individuals.

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Introduction

Diabetic mellitus (DM) is a metabolic disorder characterized by defects in action and/or secretion of insulin. It results in an elevated level of blood glucose, which leads over time to serious vascular diseases. About 422 million people worldwide have diabetes and it continues to increase globally specially in low-and middle-income countries (WHO, 2019; Misra *et al.,* 2019).

Diabetes is a multifactorial disease occurs as a result of interaction between genetic and non-genetic factors (Hivert *et al.,* 2014; Zsolt and Balogh, 2019; Ulder, 2019). Vitamin D (*VD*) has a role in a number of functions, including insulin synthesis and secretion regulation. Vitamin D's actions are determined by the activation of the vitamin D receptor (*VDR*); there by its deficiency has been linked to the onset and progression of DM. (Teegarden and Donkin, 2009; Danescu *et al.,* 2009; Garbossa and Folli, 2017; Szymczak-Pajor and Śliwińska, 2019).

The functions of *VD* are mediated via vitamin D receptor (*VDR*) gene which acts as a transcription factor regulating transcription of target genes (Uitterlinden *et al.*, 2004). Several polymorphisms have been identified in *VDR* gene including the Fok1, Taq1, BsmI and ApaI polymorphisms and their association with DM have been studied in different ethnic populations, however the results have been contradictory among different populations worldwide (Bianco *et al.*, 2004; Guo *et al.*, 2006; Mohammadnejad *et al.,* 2012; Guofeng *et al.*, 2014; Sarma *et al.*, 2018).

The gene encoding the *VDR* was found on the long arm of chromosome 12. The Fok1 polymorphism (rs2228570) located in exon 2. The substitution of thymine (T) to cytosine (C) results in ATG (methionine) changed into ACG (Threonine). The TT variant of Fok1_polymorphism give a full length VDR protein, whereas, the *VDR* CC variant result in a truncated protein (Whitfield *et al.*, 2001).

In this study we aimed to investigate a possible association of Fok1 polymorphism (rs2228570) with diabetes and its complications in Sudanese Patients

Material and Method

Sample collection and DNA extraction

A cross sectional study was carried out at Zenam Health Centre in Khartoum State. Verbal consent was obtained from patients and controls. The study was approved by the Ethical Committee of the Deanship of Scientific Research - ALNeelain University.

Three ml of blood was collected in EDTA tube from 181 of proven diabetic patients and 128 healthy controls. All participants have been confirmed with or without diabetes by an expert physician. Genomic DNA was extracted from EDTA buffy coat by the guanidine method (Tang *et al*., 2006). The quality and quantity of the DNA was checked on NanoDrop™ Spectrophotometer.

Genotyping of the FOK1 polymorphism of the *VDR* gene

The Fok1 polymorphism (rs2228570) was genotyped by PCR-RFLP method using the INTRON Biotechnology, Inc. pre-mix, followed by incubation in Fok1 restriction enzyme as describe in Table1.

Table 1: The RFLP–PCR method used to detect Fok1 polymorphism (rs2228570) of the *VDR* gene.

Primer sequence	Forward primer 5'- AGCTGGCCC TGGCACTGACTCTGCTCT-3' Reverse primer 5'- ATGGAAACACCTTGCTTCTTCTCCCTC- 3`
PCR mix contains	(i) iNtRON Biotechnology, Inc. pre-mix (ii) 1 µl of 10 pmol/µl of primer mix (iii) 3–5 µl of 50 ng/µl of template DNA (iv) Volume completed to 12.5 µl
PCR program	(i) 95ºC for 5 min for initial denaturing (ii) 30 cycles of denaturing at 95ºC for 30 sec., annealing at 60◦C, 63◦C, 66◦C for 30 sec., and extension at 72ºC for 30 sec (iii) Final extension at 72ºC for 10 min
RFLP: Restriction mix containing	(i) 3-5 µl of the PCR product (ii) 0.25 µl of 0.5 U of FOK1 restriction enzyme (New England Biolabs) (iii) 1 µl Tango buffer (iv) The mix was completed with water to 10 µl (v) The restriction mixture was incubated in 37 ºC for 3 hrs.

The homozygous mutant genotype (CC) of the *VDR*-Fok1polymorphism (rs2228570) indicated by the presence of 265 bp band. The homozygous wild type (TT) was cleaved giving a band of 196 and 69 bp. and heterozygous genotype (TC) was indicated by the presence of three fragments, 265 bp, 196 and 69 bp.

The RFLP PCR products were electrophoresed in 2% agarose gel stained with ethidium and the genotypes were visualized in gel documentation.

Statistical analysis

The analysis of data was done using SPSS software package (version 21). Statistical significance was determined at P_value<0.05. Odd ratios (ORs) were used to assess the strength of the association of the *VDR* – Fok1 polymorphism (rs10735810) with the risk of DM predisposition and its complications.

Results

Two third of the examined diabetic were females (64%) compared to 55% of the healthy controls, with no significant gender difference (P=0.08) between the two groups. (Fig.1).

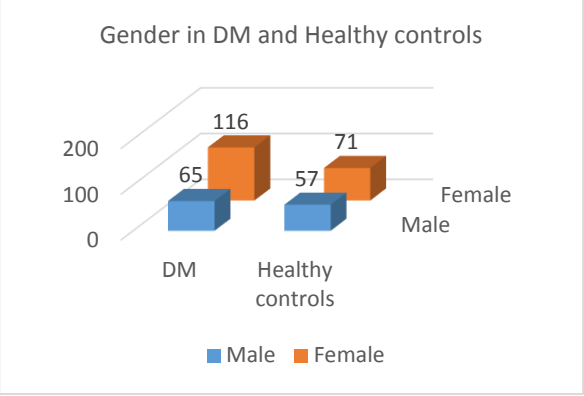

Fig.1: Shows the gender difference between Diabetes mellitus (DM) patients and healthy controls (P_Value=08).

Ninety-three of the cases were at age group of 40 years old compared to 71% of the controls of the same age group with extreme difference between cases and controls and five folds increased risk of diabetes in this age groups (P_value=0.00, OR= 5.2) (Fig.2).

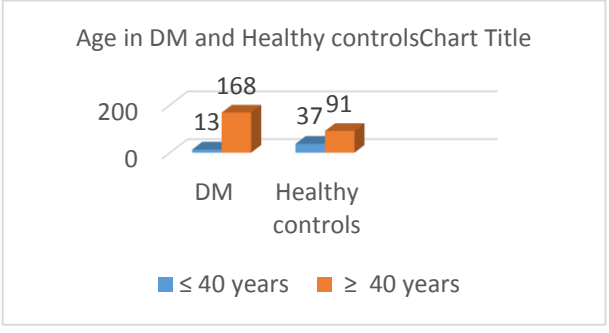

Fig.2: Shows the age difference between Diabetes mellitus (DM) patients and healthy controls (P_Value=0.00)

The diabetic septic foot (DSF), diabetic neuropathy (DNP), diabetic retinopathy (DRP); as diabetic complications; have been reported among diabetics with frequencies of 43%, 24% and 12%, respectively. Fig.3

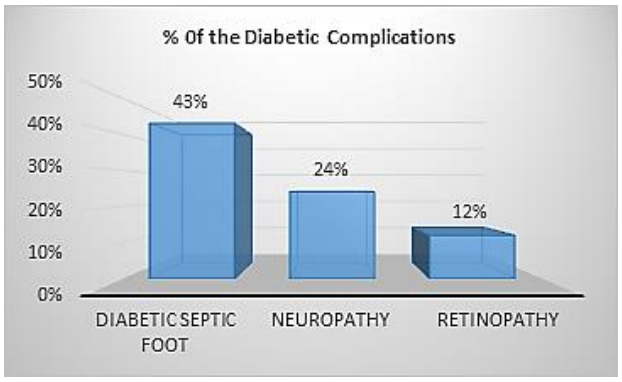

Fig. 3. Frequency of diabetic complications among diabetic Patients.

Although not all patients were able to answer with certainty the duration of the disease, our study reported for the diabetic patients who experience complication within five years or more: 80% (43/40), 77% (33/34) and 96% (21/22) for DSF, DNP, and DRP, respectively.

The genotype analysis of the Fok1 polymorphism (rs2228570) revealed a significant association with DM patients compared to the healthy controls (P-value=0.02; Table 2. The frequency of the mutant C allele was higher (80%) in DM patients as compared to the controls (71%), however, the difference was not significant. (Table 2; Fig. 4). As well as, no association between the Fok1 polymorphism (rs2228570) and the DSF, DNP, or DRP was reported (P-values 0.09, 0.21, and 0.10, respectively).

The mutant C allele was found in high frequency in DSF, DNP, and DRP (82%, 86%, and 91%, respectively. (Fig.4). There was no statistically significant difference between diabetics and DSF or diabetics and DNP; however, when comparing diabetics to DRP, the highest frequency of the C allele revealed a statistically significant difference (P_value=0.03; Table 2).

Table 2: The genotypes and allele frequency of the Fok1 (rs2228570) in diabetic patients, diabetic complications and healthy controls

Genotypes of FOK1(rs2228570)								
	DM	H/C	DM	DSF	DM	DNP	DM	DRP
Wild-type TT	2 (1 %)	5 (4 %)	0 (0 %)	2 (3 %)	2 (1 %)	0 (0. %)	2 (1 %)	0 (0 %)
Heterozygous TC	68 (38 %)	63 (49 %)	44 (42 %)	24 (31 %)	56 (41 %)	12 (28 %)	64 (40 %)	4 (18 %)
Mutant-type CC	111 (61 %)	60 (47 %)	60 (58 %)	51 (66 %)	80 (58 %)	31 (72 %)	93 (59 %)	18 (82 %)
P-value	0.02		0.09		0.21		0.10	
Alleles of FOK1 (rs2228570)								
T	20 %	29 %	21 %	18 %	22 %	14 %	21 %	9%
C	80 %	71 %	79 %	82 %	78 %	86 %	79 %	91 %
P_value	0.19		0.72		0.19		0.03	

H/C= Healthy controls, DM= Diabetic, DSF= diabetic septic foot, DNP= diabetic neuropathy, DRP= diabetic retinopathy.

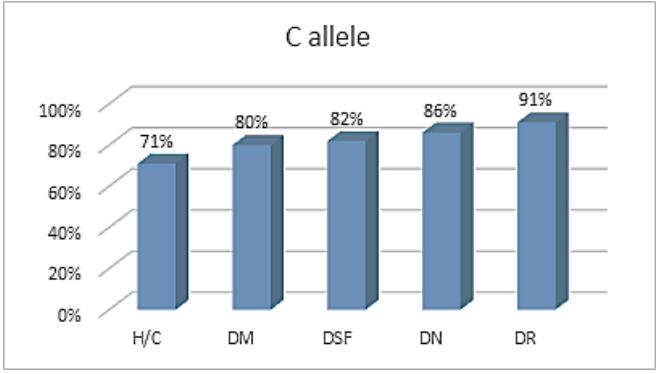

Fig.4: Percentage of the C allele among healthy controls (H/C), Diabetes mellitus (DM) and diabetic complications (DSF = diabetic septic foot, DN = diabetic neuropathy, DR = diabetic retinopathy).

Discussion

Diabetic mellitus remains one of the major public health challenges globally with high incidence and morbidity in the developing countries including Sudan; due to the genetic makeup of people in these countries, the changes in the lifestyle, coupled with an in adequate and/ or insufficient disease control in these countries (Bos and Agyemang, 2013; Awadallaa et al., 2017; Misra et al., 2019).

Previous research has found strong evidence of a genetic component to diabetes susceptibility (Hivert et al., 2014; Zsolt and Balogh, 2019). The role of VD genetics in DM has been demonstrated in a variety of ethnic groups around the world (Panierakis et al., 2009; Dilmec et al., 2010; Zhu et al., 2014; XU et al., 2014; Rahmannezhad et al., 2016; Rasoul et al., 2019).

The result showed that females represent two third of the diabetic patients (64%) and showed 93% of the patients were aged ≥ 40 years; that could be explained by the fact that females after menopause were experienced a reduced insulin sensitivity as compared with age-matched males (Hui et al., 2019).

As a result of hyperglycemia over time, diabetic patients are at risk for diabetic sores, impaired ability to fight infection, nerve injury, and poor eyesight or feeling (DPPRG, 2007; Vinik, and Ziegler, 2007; Awadallaa et al., 2017).

Diabetic septic foot and neuropathy as diabetic complications were observed with frequencies (43%, and 24%, respectively) among diabetics. Such complications represent a major concern for diabetic patients, as they adversely affect the quality of life and the long-term prognosis impose heavy economic burden to the patients and their families. This result coincided the previous studies that considered these complications as serious outcomes of diabetes in Sudan (Ahmed et al., 2016; Almobarak et al., 2017; Awadallaa et al., 2017), in Arab nations (Abdel-Motal et al., 2017) and African countries (Bos and Agyemang, 2013; Zhelong et al., 2014).

A genotyping study of the VDR-Fok1 polymorphism (rs2228570) found a significant association (P value=0.02) between the mutant genotype and DM when compared to

healthy controls. This conclusion is in line with previous study conducted in Kuwaiti and Indian populations (Bid et al., 2009; Sarma et al., 2018; Rasoul et al., 2019). However, the genotype of Fok1 polymorphism (rs2228570) did not appear to be associated to the DSF, DNP, or DRP, which contradicts the findings of Tecilazich et al. (2020).

The mutant C allele was found in high frequency among diabetics with DSF, DNP, and DRP (80, 82%, 86%, 91 respectively) suggesting that it may have a role in DM complications in Sudanese patients. Such a contribution might be explored in prospective studies to see if mutant C has a role in disease vulnerability.

Conclusion:

The Fok1 polymorphism (rs2228570) of the VDR gene was found to be strongly linked with the likelihood of acquiring diabetes in the studied Sudanese diabetic patients. The significant prevalence of the mutant C allele found in diabetic individuals with DSF, DNP, and DRP could be a predictor of illness development and consequences in Sudanese patients. Although we were unable to draw firm conclusions about the relationship between the duration of the disease and the onset of complications because we were unable to accurately determine the duration of the disease, a high frequency of diabetic complications (80 % for DSF), (77 % for DNP), and (96 % for DRP) was observed among diabetic patients within five years or more, indicating either a lack of awareness among diabetic patients about the disease and its complications, or due to insufficient information.

Recommendation

Diabetes mellitus (DM) is a complex disease, and VDR polymorphisms may contribute to the disease predisposition. Therefore, further studies of large sample targeting certain ethnic Sudanese group to avoid the population stratification, might be interesting in understanding the role of the VDR polymorphisms in the susceptibility to DM and its complications in Sudanese population.

In addition to genetic research, more emphasis should be placed on initiating an educational campaign to raise public knowledge of diabetes and its implications, as well as providing low-cost diagnostic tests and treatments to assist the Sudanese community with diabetes.

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