Polymorphism of Vitamin D Receptor (re2228570) in Sera of Coronary Artery Diseases and its Association with Various Anthropometric and Biochemical Parameters

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Abstract

Objective : Coronary artery disease is increasing and accounts for a high proportion among others diseases. In Iraq various studies have been reported that the polymorphism of vitamin D receptor VDR-*Fok I* gene like rs 2228570 are associated with CAD patients.

Aim: This study was aimed to investigate the association between the SNP of VDR *Fok I* (rs2228570) gene with various anthropometric and biochemical parameters as a risk for CAD in Iraqi population.

Methods and Materials : The current case - control study consisted of 300 samples , 150 of them were obtained from CAD patients who underwent the angiography department – heart centre – Al-Hussein Teaching Hospital, Al-Hussein Medical City / Holy Kerbala - Iraq and another 150 healthy control samples. Phenotypic data included body mass index (BMI), levels of fasting blood sugar (FBS), lipid profile, and blood urea, serum creatinine. Genotyping of rs2228570 polymorphism was carried out by PCR–RFLP method. DNA was extracted from genomic whole blood and genotyping was achieved with specific primers to amplify fragments for digestion with restriction enzymes. The enzyme *Fok I* was used for the digestion of VDR gene product followed by electrophoresis on agarose gel. Various statistical analyses were applied to analyse the data.

Result: Digestion of VDR – *Fok I* gene product (PCR-product) exhibited an amplicon size of 273 bp, when this amplicon digested with *Fok I* enzyme, it gives three genotypes indicated one (273bp), two (75 bp + 198 bp) and three (75 bp + 198 bp + 273 bp) bands for those with wild type (TT), homozygous (CC) and heterozygous (TC) genotypes respectively. Genotype frequencies of rs2228570 polymorphism were found to be consistent with Hardy–Weinberg equilibrium with allele frequencies of TT wild genotype (33.3%), TC heterozygous genotype (46.7%), and CC homozygous genotype (20%) in cases of CAD group while 66.7%, 30 % and 3.3 % for wild, heterozygous, and homozygous in the control group respectively. The homozygous genotype (CC) was significantly (OR= 7.25, CI 2.74-19.20 , P<0.001) increased the risk of CAD seven and quarter folds with respect to those of the wild type (TT) after adjustment for age, sex and BMI, while the TC genotype significantly (OR = 2.04, CI 95% ; 1.27-3.28 , P<0.001) raised the risk of CAD by two folds. Co-dominant genotypes of rs2228570 polymorphism exhibited significant association with anthropometric and biochemical parameters such as BMI, and lipid profile among patients groups as compared with control groups.

Conclusion: The obtained results improved that the gene polymorphism of VDR-*Fok I* was associated with high risk for development and progression of CAD and exhibited a significant association with increased BMI and lipid profile of coronary artery diseases of Iraqi population.

Keywords: Coronary artery disease (CAD) ; vitamin D receptor (VDR) ; *Fok I.*

Introduction

Coronary artery disease (CAD) is the most common cause of death globally and is the most prevalent cardiac disease in developed country (WHO, 2012). The morbidity and mortality and the risk factors of CAD have greatly increased and rise rapidly in latest years (He *et al.* , 2005). CAD is caused by a thrombotic occlusion of coronary arteries triggered by atherosclerotic plaque disruption, resulting in an activation of coagulation processes (Wong *et al.* , 2012). It is a complex, multifactorial, and polygenic disorder that involves an interaction between genetic and environmental factors (Dogra *et al.* , 2012). They are most traditional risk factors diet, dyslipidemia, diabetes mellitus, obesity, hypertension, inflammation, smoking, and alcohol consumption, allow the prediction of only about 50% of the absolute risk of a cardiovascular event in individual patients (Szabó and Acsády., 2011 ; Onrat *et al.* , 2012) and the remaining risk factors is attributed to genetic influences (such as single nucleotide polymorphism; SNP) play important roles in the pathogenesis and the occurrence of CAD (Sowjanya *et al.* , 2015). Genetic susceptibility may be caused by mutations and polymorphisms in a variety of genes mostly involved in blood coagulation, regulation of blood pressure, and metabolism of glucose, lipids, or homocysteine (Onrat *et al.* , 2012).

Vitamin D endocrine system is involved in a wide variety of biological processes including bone metabolism, regulation of cell proliferation and differentiation and modulation of immune responses (Uitterlinden *et al.* ,2004). The clinical role of vitamin D and VDR in the skeletal metabolism is well known (Al-Tu'ma, et. al. , 2017). Vitamin D receptor (VDR) is a protein of 427 amino acids, with molecular mass of approximately 48 kDa .It is adirect regulator of gene transcription of a number of hormone responsive genes and plays an important role in the vitamin D pathway, VDR was belonging tothe nuclear receptor family.It is present on the long arm of chromosome12 (12q12-14) (Buyru *et al* ., 2003). Recent studies have well-characterized four VDR polymorphisms *Fok1*, *Bsm1*, *Apa1* and *Taq1* and these polymorphisms within the VDR gene may potentially influence the vitamin D expression and the stability of VDR mRNA (Uitterlinden *et al* .,2004). *Fok I* is located in exon 2 at the 51 coding region (Tamakoshi *et al*., 2003). Studies have demonstrated that VDR are present in the aortic endothelial, vascular smooth muscle cells etc. VDR polymorphisms may influence the susceptibility to CAD (Xiang *et al* ., 2017 ; Sowjanya *et al* ., 2015).

This study was aimed to investigate the association of SNP of VDR *Fok I* (rs2227580) with risk for CAD in Iraqi population and to show the impact of (rs2227580) on anthropometric and some biochemical parameters by analysis of phenotypic data connected to the genotypes of VDR (rs2227580) polymorphism.

Materials and Methods

This (case – control) study consist of 300 samples of both gender, 150 of them were randomly selected as normal healthy control group ; their ages ranged between 40-70 years with a mean of BMI (23.7 ± 0.9) kg/m^2 , and another 150 subjects with CAD who underwent to the angiography department in the cardiology department of Al-Hussein Medical City in Karbala /Iraq were selected as the patients group. The patient's age ranged between 40-70 year with a mean of BMI (29.2 ± 3.1). Subjects with any known endocrine disorders that effect on vitamin D metabolism were excluded. The current study was approved by the institutional ethics committee. After taking an informed written consent, 4 ml of blood was drawn by vein puncture from all individuals participated in this study. The blood was divided into two parts: The first part was used for gene analysis. It included one ml of blood collected in EDTA containing tube and used for DNA extraction , then were analyzed directly to obtain high purity of DNA. The second part included three ml of blood placed in serum tube were stored at -20°c until analysis. The EDTA samples were analyzed by PCR -RFLP method, where serum sample used for measurement of total cholesterol, LDL-C, HDL-C, and TG by using commercially available kits (colorimetric assay, Biolabs , France).The study was managed throughout the period since November 2016 to October 2017. The research was done in the laboratories of biochemistry department, College of Medicine / University of Kerbala.

Genomic DNA was extracted from peripheral blood using the Relia Prep™ Blood g DNA Mini prep System kit (Promega) as per the manufacturer's protocol. Amplification of the gene of VDR was performed using primers described previously by (Mishra et al ., 2013), as shown in table -1-.

Table 1: Primer sequences, annealing temperature(AT) and product size of vitamin D receptor

Vitamin D Receptor Gene T↔C (Met 1 Thr) SNP
Forward primer 5′- GAT GCC AGC TGG CCC TGG CAC TG-3′
Reverse primer ′5-ATG GAA ACA CCT TGC TTC TTC TCC 3′
AT 60 °C
Product size 273 bp

Reaction was setup with standard PCR reagents in a 25 µl reaction volume containing 5 µl DNA sample, 12.5 µl mater mix, 1.5 µl forward primer, 1.5 µl reverse primer, 4.5 µl nuclease free water. A directly above limitations providing a 25 µL reaction volume that additional in 500µl PCR tube at 25 °C then, centrifuged for 30 seconds at 2000 xg in a micro centrifuge for mixing the sample tubes and then placed in thermocycler. The PCR reactions were carried out as follows Table -2-.

Table 2 : Thermocycler program for PCR amplification of VDR-*FokI* gene T↔C (Met 1 Thr).

Type of Cycle	**Temperature, °C**	**Time , minute**	**No. of Cycles**
Initial denaturation	94	4 min	1
Denaturation	94	1 min	30 x
Annealing	60	1 min	
Extension	72	1 min	
Final extension	72	4 min	1
Total time : 2 hours and 4 mints			

PCR amplification was confirmed by 2% agarose gel electrophoresis. 100bp DNA Leader (Bioneer , south Korea) was used to confirm the amplicon size and the gel was visualized in the gel documentation system. *Fok I* genotypes were analysed by using with 5 unit of *Fok I* restriction enzyme and the reaction buffer and incubated at 37°C for 1hours; 10 µl micropipette of the digested reaction mixture was then loaded into 2% agarose gel containing ethidium bromide. The wild genotype (TT) lacked a Fok-I site and showed only one band of 273 bp. The homozygote genotype (CC) generated two fragments of 75 bp and198 bp. The heterozygote displayed three fragments of 273, 75 bp and 198 bp, designated as (TC). Genotype quality and validation was done by performing repeated assay on 20% of the samples selected at random. Samples with known genotypes were included in the reaction to confirm the genotypes.

The continuous data were expressed as mean ±SD , T test and the ANOVA table will be used for calculating Probability by using the statistical analysis program IBM SPSS version 24 (SPSS Inc., Chicago, IL). The portable statistical program Win Pepi version 11.65 used for calculating Pearson's chi-square, Fischer exact probability and odd ratio which used to express the significance between the studied groups for polymorphisms and related parameters. Categorical data (genotypes and alleles) were expressed as frequency and calculated by Hardy–Weinberg equilibrium (HWE).

Result:

A total of 150 CAD patients, their ages ranged between (40 -70) year and the observed (mean ± SD) was (57.3 ±8.3) year. The other 150 sample were controls with age ranged between (40-70) years and the (mean ± SD) was (57.5± 8.0) years and the association of VDR gene polymorphism of *Fok I* with CAD was evaluated.

The distribution of VDR *Fok I* genotype shows in Table -3- . Among 150 of cases CC homozygous genotype was observed in 30, TC Heterozygous genotyping in 70 and TT Wiled genotyping in 50 subjects. In controls, among the 150 subjects 5 were CC, 45 were TC and 100 were TT genotypes. 95% CI and odds ratio were calculated, which show significant association with CAD.

Table 3 :The distribution of VDR *Fok I* genotype among patients and control groups.

Genotype	Patients No.	%	Control No.	%	OR	CI 95%	P-value
(Met 1 Thr) T ⟶ C	150		150				
TT	50	33.3	100	66.7	0.25	0.15-0.40	P<0.001
TC	70	46.7	45	30	2.04	1.27-3.28	P<0.001
CC	30	20	5	3.3	7.25	2.74-19.20	P<0.001

* P<0.001 : Statistically significant ; OR : Odd ratio ; CI : Confidence interval

Table -4- shows the allele frequency in cases and controls. The odds ratio is significant about twice for the development of CAD in TC the genotypes and seven and quarter in CC the genotype compared to TT genotype. Fig .1 shows the TT ,TC,CC genotype band on 2% ethidium bromide gel ,while Table -5- shows BMI and lipid profile of CAD and control subjects according to VDR gene polymorphism (rs2228570) genotype.

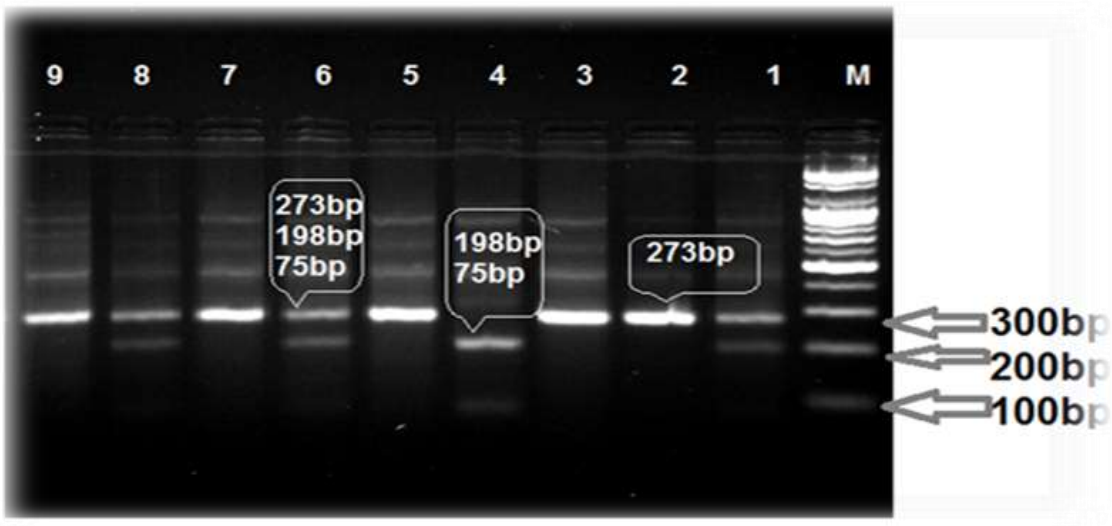

Fig.1. Gel electrophoretic diagram showing the genotype bands (TT genotype –single band with 273bp, CC genotype –two band with (198bp , 75 bp) and TC genotype –three band with (273bp,198bp,75b)

Table 4 : Fok I Allele frequency by Hardy –Weinberg equilibrium (HWE) in CAD cases and control subjects

Genotype	No.	Allele frequencies	HW-observed frequency	Percentage %	HW-expected frequency	Percentage %	X2	P-value HWE
Cases								
TT	50	T: 0.57	50	33.3	48.2	32.1	0.37	P>0.05
TC	70	C: 0.43	70	46.7	73.7	49.1		
CC	30		30	20	28.1	18.8		
Control		T:0.82						
TT	100	C:0.18	100	66.7	100.1	66.8	0.0005	P>0.05
TC	45		45	30	44.9	29.9		
CC	5		5	3.3	5.0	3.3		

X_2: Chi square ; P>0.05 statistically significant by Hardy –Weinberg Equilibrium (HWE); **C:cytosine ; T : thymine.**

Table -5- Shows BMI and lipid profile of CAD and control groups according to VDR gene.

Genotypes	BMI (kg/m^2)			Triglyceride , mg/100 ml		
	Control	Patients	P-value	Control	Patients	P-value
	Mean ±SD	Mean ± SD		Mean ±SD	Mean ±SD	
TT	23.8±0.7	28.5±2.9	P<0.001	143.4±36.2	240.2±31.9	P<0.001
TC	23.5±0.8	29.2±3.6	P<0.001	144.9±34.6	242.6±27.5	P<0.001
CC	23.4±0.5	30.1±2.7	P<0.001	161.6±50.6	249.1±15.4	P<0.001
	HDL-C, mg/100 ml			Total Cholesterol, mg/100 ml		
	Control	Patients	P-value	Control	Patients	P-value
	Mean ±SD	Mean ±SD		Mean ±SD	Mean ±SD	
TT	51.8±6.6	43.4±8.3	P<0.001	118.3±20.1	160.9±23.0	P<0.001
TC	49.6±2.3	40±7.7	P<0.001	120.3±25.4	161.8±3.7	P<0.001
CC	48.5±8.9	38.4±8.3	P<0.001	128±25.8	162.8±21.3	P<0.001
	VLDL-C, mg/100 ml			LDL-C, mg/100 ml		
	Control	Patients	P-value	control	Patients	P-value
	Mean ±SD	Mean ±SD		Mean ±SD	Mean ±SD	
TT	23.9±4.1	32.4±4. 6	P<0.001	68.8±31.5	165.9±31.3	P<0.001
TC	24.3±4.8	32.2±4. 7	P<0.001	72.1±39.1	170.7±28.6	P<0.001
CC	25.8±5.2	32.9±4. 8	P<0.001	86.2±44.7	174.0±18.4	P<0.001

*** P<0.001 statistically significant; C :cytosine ;T :thymine**

Discussion

Coronary artery disease is a common heart disease with global health problems. Different studies detected vitamin d has a positively impact on cardiovascular health (Hossein-nezhad and Holick ., 2013 ; Al-Tu'ma and Yosuf, 2015)) . Vitamin D is initially metabolized to the intermediate compound 25-hydroxyvitamin D in the liver which subsequently binds to the intracellular receptors to regulate gene expression. Furthermore, a low vitamin D level is associated with increased cardiovascular morbidity and mortality in the general population (Melamed *et al* ., 2008).vitamin D was linked with the nuclear receptor VDR for the whole genome action. VDR gene has various polymorphisms in coding region .They were informed which are

Fok I located in starting codon of exon 2 in restriction site while *BsmI, ApaI, TaqI* those located in exons 8, 9, 10 respectively. The importance of *Fok I* polymorphism because It was located in exon 2 which is the start codon translation site at 5 end of VDR gene and the change of sequence from T to C in the initiation codon that clues to alteration the codon sequence from ATG to ACG. Thus, when the C variant is present, an alternative start site is used producing a protein with different sizes. Variant T was longer than variant C by three amino acids which are methionine, glutamic acid and alanine that had been revealed functionally less effective such as described by various studies (Abbas *et al* ., 2008 ; Whitfield *et al* ., 2001 ; Uitterlinden *et al* .,2004) . Most of experiments conducted so far point to the fact that the protein (424 AA) of short form is more active than that of long form (427 AA) in terms of its transactivation activity as a transcription factor. However, it seems to be gene-specific and cell type-specific. Thus, a certain genes and cell types will be more sensitive to the polymorphism than others (Ingles *et al* .,1997) It is clear from the present study that reduced number of patients group with wild TT genotype polymorphism by 33.3 when compared with 66.7 % found in healthy control group. Also, the present study demonstrated that VDR- FOKI gene polymorphism was linked with the danger of expanding coronary artery disease .There were elevated risk of coronary artery disease in patients who carried out heterozygous alleles TC by approximately two times and in homozygous CC alleles genotype by about seven and quarter times when compared with patients who carried out common homozygous allele TT genotype after adjustment for age and BMI. Such observations strongly suggested a role of VDR gene polymorphism (rs2228570) in the pathogenesis of CAD in Iraqi patient. Results of this study are in covenant with the results one Arab populations Egyptians (Abu el Maaly *et al* .,2016) .They are also an agreement with data of studies populations of Han Chinese(He and Wang ., 2015), Hossein-nezhad et al reported the role of vitamin D through VDR –*Fok* rs 2228570 on coronary artery disease in Iranian population (Hossein-nezhad and Holick ., 2013), But Pan et al reported the Fok1 polymorphism was not found to be associated with CAD incidence in Chinese (Pan ., 2009). Sowjanya etal reported no significant association of VDR fok I polymorphism with CAD in south Indian population (Sowjanya *et al* .,2015) also to show the impact of rs(2228570) on anthropometric and biochemical parameters by analysis of phenotypic data connected to the genotypes (TT,TC,CC) of VDR (rs2228570) polymorphism .The difference in the occurrence of genotypes in pointed out a remarkable observation which is the significant variation of lipid profile for patients and control group among the three groups (TT,TC,CC) . The lowest HDL level was found in carriers of the CC genotype, while the highest values observed in those of TT genotype. Inversely, highest cholesterol, TG , LDL-C,VLDL-C magnitudes were noticed in carriers of CC carriers and lowest values were found in those of TT carriers. As regards, the inverse relationship of HDL-C levels with rs 2228570 polymorphism and for our knowledge, the investigation at which such observation was reported by Schuch *et al*.,2013 and Filus *et al*.,2008) which suggest that two major VDR gene polymorphisms (*BsmI and FokI*) seemed to influence BMI, insulin resistance, and serum HDL cholesterol .The current finding are a likely method to study changes of lipid profile levels with respect to various VDR gene polymorphism as well as it could be considered in early protection and management of cardiovascular diseases. Changes of BMI values are indicated to be significant among comparison of the three groups of genotypes for patients and controls , but it is very difficult to speculate and obtain correct decision, since changes are not stratified. Several authors have investigated the impact of rs 2228570 polymorphism on BMI values (Schuch *et al* .,2013 ; Filus *et al* .,2008). They suggests that the VDR gene polymorphisms appear to be associated with metabolic syndrome (dyslipidemia, high blood pressure, and high cholesterol) . Metabolic syndrome considers the major risk factors for development of cardiovascular disease (Zhou *et al* .,2008). In conclusion, our findings support the hypothesis that VDR *FokI*- CC genotype may predict associated with high risk for development and progression of CAD In Iraqi population . and by using a common VDR polymorphism data

suggests they may influence an lipid profile and BMI in our population.

Conclusion

The obtained results improved that the gene polymorphism of VDR-*Fok I* was associated with high risk for development and progression of CAD and exhibited a significant association with increased BMI and lipid profile of coronary artery diseases of Iraqi population.

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