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Regular paper

Genetic association between vitamin D receptor gene and Saudi patients confirmed with Familial Hypercholesterolemia

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Introduction: Familial Hypercholesterolemia (FH) is a common condition caused by inherited genetic abnormalities. Inadequate clearance of the circulating low-density lipoproteins (LDL) is the primary cause of the excessive concentrations of LDL seen in FH patients. The relation with vitamin D deficiency and vitamin D receptor (VDR) gene is well documented in the Saudi Arabia. Aim: The aim of this study was to investigate the role of molecular analysis studied between FH patients and fours polymorphisms associated with VDR gene in Saudi Population. Methods: In this case-control study, 120 patients were selected, and 50 patients were confirmed as FH and 70 subjects were confirmed as healthy controls. Genotyping was performed with polymerase chain reaction followed by restriction fragment length polymorphism analysis using ApaI, BsmI, TaqI and FokI polymorphisms in the VDR gene. Results: The current study results confirmed no association between clinical characteristics studied between FH cases and controls ($p>0.05$). Hardy Weinberg Equilibrium analysis was present in ApaI and FokI polymorphisms ($p<0.05$). Only ApaI (C *vs* A: OR-15.1 (95% CI:5.78-39.41); $p<0.001$; AC+CC *vs* AA: OR-6.59 (95% CI:2.42-17.95); $p=0.0006$) and BsmI (G *vs* A: OR-2.88 (95% CI:1.54-5.38); $p=0.0006$ and AG+GG *vs* AA: OR-3.79 (95% CI:1.72-8.35); $p=0.0007$) polymorphisms showed both allele and genotype association between FH patients and controls. ANOVA analysis confirmed that TG levels were associated ($p=0.02$) with combination of heterozygous and homozygous genotypes present in all four polymorphisms studied in this population. Conclusion: ApaI and BsmI polymorphisms in the VDR gene showed association with FH patients in the Saudi Population.

Keywords: Familial Hypercholesterolemia, low-density lipoproteins, vitamin D

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Abbreviations: BMI, Body Mass Index; DLCN, Dutch lipid clinical network; FH, Familial Hypercholesterolemia; HoFH, Homozygous Familial Hyper-cholesterolemia; HWE, Hardy-Weinberg equilibrium; LDL, low-density lipoproteins; PCR, Polymerase Chain Reaction; TC, total choles-terol; TG, Triglycerides; VDR, vitamin D receptor

INTRODUCTION

Familial Hypercholesterolemia (FH) is defined as a common disorder caused due to inherited genetic defects which is responsible for elevated LDLc levels due to inadequate clearance of the circulating LDL (Hori *et al.*, 2023). The definition of FH is described as elevated levels of LDLc which describes in time the development of atherosclerosis process in the arteries with subsequent high risk of cardiovascular diseases (Maștaleru *et al.*, 2022). Extremely high levels of LDL-c are the result of homozygous familial hypercholesterolemia (HoFH), a severe and rare form of FH. If ignored, HoFH can cause atherosclerotic cardiovascular disease in the early decades of life. An estimated 1 in 300 000–360 000 people in the United States have HoFH. Isolated groups and those experiencing the founder effect also have a higher incidence (Kayikcioglu & Tokgozoglu, 2023). Clinical scoring systems for the diagnosis of FH are widely used and approved, and numerous sets of criteria have been created, encompassing clinical, biochemical, and genetic factors. The most commonly used criteria are Simon Broome, MedPed and Dutch lipid clinical network (DLCN), the last one being used predominantly in the European countries (Huangfu *et al.*, 2023; Todorovova *et al.*, 2023). Some patients have unusual alleles in the minor genes *APOA5, ABCG5, ABCG8, LIPA,* and *STAP1*, whereas the vast majority have mutations in the classically related FH genes *LDLR, APOB*, or *PCSK9*. Interestingly, many FH cases either demonstrate a mixture and accumulation of numerous common variations or have no known genetic etiology. In addition, inflammation, vascular tone, cellular proliferation, and other unknown processes account for 80% of common DNA variations that predispose an individual to coronary artery disease (Ruiz-Pesini *et al.*, 2023). Previous meta-analysis estimates the worldwide frequency of heterozygous FH to be 1:313 (Beheshti *et al.*, 2020). However, the estimated heterozygous FH prevalence in the Arabian Gulf region is 1:112, which is around three times the predicted incidence worldwide (Alhabib *et al.*, 2021). Based on the incidence of 1:200–500, the estimated number of HeFH patients in Saudi Arabia ranges from 63 485 to 158 712. The lack of genetic screening programs and governmental data makes it difficult to determine the true FH incidence in Saudi Arabia and the Middle East (Alzahrani *et al.*, 2020). In Saudi Arabia, information on the genetics of FH is extremely scarce. The literature revealed that 21 variations were linked to *LDLR, APOB* and *PCSK9* genes and 80% of these variants were predicted to influence the *LDLR* gene, and c.1332dup (p.D445*) and c.2026delG (p. G676Afs*33) mutations were confirmed to be novel variants in the *LDLR* gene (Al-Allaf *et al.*, 2014).

In Saudi Arabia, the main causes of vitamin D deficiency are cultural practices, climate, genetic disposition, and skin color. Furthermore, high temperatures are predisposed to this deficiency (Al-Alyani *et al.*, 2018). It was well-established that Vitamin D will be converting into 25-hydroxyvitamin D in liver and kidney will be converting into 1,25-dihydroxyvitamin D3, which has a biological purpose (Bikle, 2014). Expression of *VDR* in mel-

anocytes allows it to control melanin production. Vitamin D is effective at treating a variety of skin conditions in humans (Tang *et al*., 2018). The vitamin D receptor (VDR) is a nuclear steroid receptor that functions as a DNA-binding transcription factor. Vitamin D activity and metabolic concentrations are both affected by polymorphisms in the *VDR* gene (Guo *et al*., 2023). The *VDR* gene is present on chromosome 12p13, which has variants in exons 2 and 3 for the enzymes *ApaI, BsmI*, and *TaqI*, and in exon 2 for the enzyme *FokI*. The restriction enzymes for *VDR* gene polymorphisms can be used to analyze the gene's many polymorphic sites in its untranslated region; which influences the functional stability of the transcript. Alleles with and without certain restriction sites are designated A-a, B-b, T-t, and F-f, respectively (Ghada Bin Saif & Imran Ali Khan, 2022). Numerous studies on the *VDR* gene in various human diseases have been conducted in Saudi Arabia, but no studies on the *VDR* gene and FH have been documented. The aim of this study was to investigate the role of *VDR* gene polymorphism studies in FH patients in the Saudi Arabia.

MATERIALS AND METHODS

FH subjects

A case-control study was conducted from King Khalid University Hospital in Riyadh city. The FH cases were confirmed based on Dutch group protocol (Alharbi *et al*., 2015) which is an inclusion criteria. The FH subjects without Dutch group criteria is considered to be excluded. The Dutch group criteria is defined as the diagnosis of FH based on clinical, genetic and family history. However, in our study, clinicians have confirmed the FH cases based on the previous study (Alharbi *et al*., 2015).

Healthy controls were selected based on normal levels of lipid profile. In this study, 70 FH cases and 50 healthy controls were recruited based on signing of patient informed consent form. This study was performed based on Declaration of Helsinki and ethical grant was approved within the hospital premises (E-19-1176). All patients (n=120) have signed the informed consent form to enroll in this study.

BMI details

Body Mass Index (BMI) was calculated based on Alshammary *et al* studies using body weight and height (Alshammary & Khan, 2021).

Sample collection

In this study, 5ml of blood was collected from 120 participants. Peripheral blood was separated into 3ml serum sample and 2ml in EDTA tube. Serum blood was used to measure the lipid profile parameters such as LDLc, HDLc, total cholesterol (TC) and triglycerides (TG). Lipid profile was performed based on the previous study (Batais *et al*., 2019).

Molecular analysis

EDTA sample was used for extraction of genomic DNA using DNA isolation kit. Extracted genomic DNA was used to quantify the DNA quality using NanoDrop spectrophotometer. VDR gene primers were adapted from the recently published article (Ghada Bin Saif & Imran Ali Khan, 2022) and all 4 single nucleotide polymorphisms were selected. The primer sequence of four polymorphisms were shown below:

(ApaI/TaqI) F: AGAGCATGGACAGGGAGCAAG and R: GCAACTCCTCATG GCTGAGGTCTCA; (BsmI) F: CAACCAAGACTACAACCGCGTCAGTGA and R: AACCAGCGGAAGAGGTCAAGGG; (FokI) F: AACCAGCGGAAGAGGTCAAGGG and R: ATGGAAACACCTTGCTTCTTCTCCCTC

Next, Polymerase Chain Reaction (PCR) was amplified with 4 polymorphisms present in the *VDR* gene. The initial denaturation took place for 10 mins at 95°C, denaturation at 95°C–30s, annealing took place at different temperatures for 4 polymorphisms, extension took place at 72°C–45s and final extension at 72°C–10 mins. The conditions of thermal cycler were followed for 35 cycles. After completion of amplification, PCR products were run on 2% of agarose gel stained with ethidium bromide and then restriction fragment length polymorphism analysis was performed using specific restriction enzymes as discussed in Table 1. Digestion was performed based on previous study (Alharbi *et al*., 2017) for 24 hours. All types of PCR products were run on 2% agarose gel.

Statistical analysis

The visible data is regularly distributed and reported as mean ± S.D.; this study was conducted as a frequency-based analysis. Chi-square analysis was used to identify statistically significant differences between the case and control groups. Goodness-of-fit analysis was used to establish Hardy-Weinberg equilibrium (HWE). The frequencies of each genotype and allele were computed. Genotype and allelic frequencies comparisons were made between healthy controls and vitiligo patients, and other genetic models. This study was determined as the odds ratio and its 95% CI. In this study, a one-way Anova analysis (Khan *et al*., 2019) was used in Table 4. However, *p*-values below 0.05 were regarded as statistically significant ($p<0.05$).

RESULTS

In this research, a total of 70 FH cases and 50 healthy control subjects were enrolled. The FH patients were clinically diagnosed based on Dutch group criteria and equally 50% of males and females were selected in both FH cases and controls. The mean age for both study groups were in between 51.98±10.61 in controls

Table 1. List of VDR gene polymorphism and restriction enzymes used in this study

Rs number	PCR products	Annealing temperature	Restriction enzymes	Restriction time	Temperature for digestion	Digested products
rs79785232	746bp	66°C	ApaI	18 hours	37°C	746/529/217bp
rs1544410	872bp	66°C	BsmI	18 hours	37°C	872/701/171bp
rs731236	746bp	66°C	TaqI	18 hours	37°C	427/293/252/201/169/92bp
rs2228570	267bp	66°C	FokI	18 hours	37°C	267/193/70bp

Table 2. Anthropometric measurements between FH cases and control subjects

S. No	Patients' measurements/Values	FH (n=70)	Controls (n=50)	*T*-tests
1	Age (years)	52.47±10.51	51.98±10.61	0.80
2	Gender (Female: Male)	35:35	25:25	0.99
3	Weight (kgs)	73.75±9.15	73.15±9.21	0.35
4	Height (cms)	165.56±7.47	165.35±7.58	0.15
5	BMI (kg/m^2)	26.84±2.49	26.81±2.53	0.06
6	TG (mmol/L)	2.02±1.34	1.67±0.87	0.11
7	TC (mmol/L)	5.29±0.94	5.01±0.98	0.11
8	HDLc (mmol/L)	0.68±0.21	0.63±0.24	0.22
9	LDLc (mmol/L)	3.76±0.83	3.80±0.92	0.80

Table 3. HWE analysis was studied in POLYMORPHISMS s in the *VDR* gene

Polymorphisms	Minor allele	VAF	χ^2	HW *p*-value
rs79785232	C	0.05	0.13	0.70
rs1544410	G	0.17	6.55	0.01
rs731236	C	0.27	6.83	0.008
rs2228570	T	0.13	1.11	0.29

P-value indicates one degree of freedom (if $p<0.05$ indicates non-consistent with HWE; VAR – Variable Allele Frequencies)

Table 4. Genotype and allele frequencies between FH cases and controls in the *VDR* gene

VDR-Genotypes	Controls (n=50)	FH cases (n=70)	ORs (95%CI); *p*-values
AA (ApaI)	45 (90%)	39 (55.7%)	Reference
AC	05 (10%)	31 (44.3%)	OR-7.15 (95% CI: 2.53-20.18); $p=0.0005$
CC*	00 (0%)	00 (0%)	OR-1.15 (95% CI: 0.02-59.39); $p=0.94$*
AC+CC *vs* AA*	05 (10%)	31 (44.3%)	OR-6.59 (95% CI: 2.42-17.95); $p=0.0006$
A	95 (0.95)	78 (0.56)	Reference
C	05 (0.05)	62 (0.44)	OR-15.1 (95% CI: 5.78-39.41); $p<0.001$
AA (BsmI)	37 (74%)	30 (42.9%)	Reference
AG	09 (18%)	28 (40%)	OR-3.83 (95% CI: 1.57-9.36); $p=0.002$
GG	04 (08%)	12 (17.1%)	OR-3.7 (95% CI: 1.08-12.65); $p=0.02$
AG+GG *vs* AA	13 (26%)	40 (57.1%)	OR-3.79 (95% CI: 1.72-8.35); $p=0.0007$
A	83 (0.83)	88 (0.63)	Reference
G	17 (0.17)	52 (0.37)	OR-2.88 (95% CI: 1.54-5.38); $p=0.0006$
TT (TaqI)	23 (46%)	46 (65.7%)	Reference
TC	27 (54%)	24 (34.3%)	OR-0.44 (95% CI: 0.21-0.93); $p=0.03$
CC*	00 (0%)	00 (0%)	OR-0.51 (95% CI: 0.009-26.27); $p=0.73$
TC+CC *vs* TT*	27 (54%)	24 (34.3%)	OR-0.45 (95% CI: 0.21-0.94); $p=0.03$
T	73 (0.73)	116 (0.83)	Reference
C	27 (0.27)	24 (0.17)	OR-0.55 (95% CI: 0.31-1.05); $p=0.06$
CC (FokI)	37 (74%)	53 (75.7%)	Reference
CT	13 (26%)	17 (24.3%)	OR-0.91 (95% CI: 0.39-2.11); $p=0.83$
TT*	00 (0%)	00 (0%)	OR-0.71 (95% CI: 0.01-36.11); $p=0.85$
CT+TT *vs* CC*	13 (26%)	17 (24.3%)	OR-0.90 (95% CI: 0.39-2.07); $p=0.81$
C	87 (0.87)	123 (0.88)	Reference
T	13 (0.13)	17 (0.12)	OR-0.92 (95% CI: 0.42-2.01); $p=0.84$

Table 5. Anova Analysis Performed between Heterozygous and Variant genotypes in FH cases with BMI and lipid profile

	rs79785232 (AC=31)	rs1544410 (AG+GG=40)	rs731236 (TC=24)	rs2228570 (CT=17)	*p*-value
BMI (kg/m²)	27.32±2.29	27.08±2.61	26.54±2.69	27.76±1.56	0.12
TG (mmol/L)	2.32±1.63	2.01±1.18	1.91±1.16	2.31±1.96	0.02
TC (mmol/L)	5.34±0.84	5.41±0.91	5.62±0.90	5.42±1.16	0.48
HDLc (mmol/L)	0.67±0.18	0.68±0.20	0.69±0.19	0.65±0.25	0.46
LDLc (mmol/L)	3.74±0.90	3.83±0.82	3.72±0.88	3.90±0.81	0.93

and 52.74±10.51 in FH cases (p=0.80). Both cases, FH (26.84+2.49) and controls (26.81+2.53), were found to be overweight (p=0.06) and mean levels of weight was found to be an average of 73kg in both groups (p=0.35). None of the parameters of lipid profile (TG/TC=0.11; HDLC=0.22 and LDLC=0.80) were associated when compared between FH group and control. Table 2 defines the general characteristics used for FH group and control group.

The polymorphic site studied in VDR gene was in Hardy Weinberg Equilibrium in the whole sample. In this study, p-value was measured with one degree of freedom in which p<0.05 is considered as non-consistent. In this study, ApaI (p=0.70 and X^2=0.13) and FokI (p=0.008 and X^2=6.83) were associated whereas in other polymorphisms such as BsmI (p=0.01 and X^2=6.55) and TaqI were non-significant. Table 3 consists of HWE analysis details.

Table 4 presents the group of polymorphisms present in the *VDR* gene in FH cases and controls. The ApaI polymorphism was strongly associated with allele (OR-15.1 (95% CI: 5.78-39.41); p<0.001), genotypes (AC *vs* AA; OR-7.15 (95% CI: 2.53-20.18); p=0.0005) and dominant model (OR-6.59 (95% CI: 2.42-17.95); p=0.0006). A similar association was found in BsmI polymorphism (G *vs* A: OR-2.88 (95% CI: 1.54-5.38); p=0.0006; AG *vs* AA: OR-3.83 (95% CI: 1.57-9.36); p=0.002 and AG+GG *vs* AA: OR-3.79 (95% CI: 1.72-8.35); p=0.0007). A negative impact was confirmed in both TaqI (C *vs* T: OR-3.79 (95% CI: 1.72-8.35); p=0.0007; TC *vs* TT: OR-0.44 (95% CI: 0.21-0.93); p=0.03 and TC+CC *vs* TT: OR-0.45 (95% CI: 0.21-0.94); p=0.03) and FokI (T *vs* C: OR-0.92 (95% CI: 0.42-2.01); p=0.84; CT *vs* CC: OR-0.91 (95% CI: 0.39-2.11); p=0.83 and CT+TT *vs* CC: OR-0.90 (95% CI: 0.39-2.07); p=0.81). Yates correction couldn't document the association in any of genotypes or allele in FH groups.

Anova analysis was confirmed in TG group (p=0.02) in the lipid profile and BMI parameters. TC (p=0.48), HDLC (p=0.46), LDLC (p=0.93) and BMI (p=0.12) were not associated when compared with heterozygous and homozygous mutants in ApaI, BsmI, TaqI and FokI polymorphisms in *VDR* gene. Table 5 shows the ANOVA analysis within FH heterozygous and homozygous mutants in the *VDR* gene.

DISCUSSION

The hypercholesterolemia that runs in families is called familial hypercholesterolemia, and it's inherited in an autosomal dominant pattern. Patients are typically heterozygous, meaning they carry only one copy of the genetic mutation. Homozygosity occurs exceedingly infrequently when a patient gets an erroneous gene from both parents; having homozygous FH results in extraordinarily high blood cholesterol levels. LDLR gene mutations are frequently responsible. Gain-of-function mutations in *ApoB* and *PCSK9* genes, among others, have been reported (Pejic & Lee, 2006). Patients with FH have elevated LDL-c blood levels, which increases the risk of coronary artery disease and heart attack in the future (Alharbi *et al.*, 2015). The relationship between cholesterol and vitamin D is that human skin cells require cholesterol in order to produce vitamin D when exposed to sunlight. The initial phase requires cholesterol, but vitamin D is converted further in the liver and kidneys. Increased risk of cardiovascular disease (CVD) has been associated with low serum 25-hydroxyvitamin D [25(OH)D] levels, which plays an important part in the onset of osteoporosis, but it has also been connected to other health problems. Diabetes (type 1 and 2), multiple sclerosis, autoimmune, and viral diseases have all been linked to vitamin D deficiency. The relation between Vitamin D and cholesterol is connected with 7DHC, making the influence of vitamin D status on blood lipids particularly intriguing, despite conflicting findings from earlier studies (Gumus *et al.*, 2023; Hong, 2022). Documented studies in vitamin D deficiency have been discovered as a potential risk factor for CVD, either independently or in conjunction with other cardiovascular risk factors such as diabetes, hypertension, or obesity (Burgess & Gill, 2022; Norman & Powell, 2014; Pilz *et al.*, 2016). The main cause of vitamin D deficiency include a lack of sun exposure, a sedentary lifestyle, being overweight, having type 2 diabetes, lower HDLC level, being over age, having dark skin, living far from the equator, experiencing winter, being a smoker, being exposed to secondhand smoke, having impaired absorption due to renal and liver disease, and taking certain medications (Mozos & Marginean, 2015).

The aim of this study was to investigate the role of *VDR* gene polymorphism in diagnosed FH patients in the Saudi population. The present study results confirmed that ApaI and FokI polymorphisms were found to be in HWE analysis. Only ApaI and BsmI polymorphisms showed both allele and genotype association between FH patients and controls (p<0.05). In our study, ApaI (AC *vs* AA: p=0.0005, AC+CC *vs* AA: p=0.0006 and C *vs* A: p<0.001) and BsmI (AG *vs* AA: p=0.002, GG *vs* AA: p=0.02, AG+GG *vs* AA: p=0.0007 and G *vs* A: p=0.0006) polymorphism was associated when compared between FH cases and controls. ANOVA analysis confirmed that TG levels were associated with combination of heterozygous and homozygous genotypes present in all four polymorphisms studied in this population. The first step was measuring the cholesterol and vitamin D levels of healthy women. As a result of Gemfibrozil and Atorvastatin's ability to inhibit HMG-CoA reductase production, blood cholesterol levels have dropped dramatically in the second phase. Within this population, vitamin D levels also went down. Women who took medication for vitamin D, on the other hand, had a low vitamin D level (Han *et al.*, 2021). Other similar studies have been documented between vitamin D and

cholesterol related diseases. One of the Spanish studies measured vitamin D (25(OH)D) levels in hypercholesterolemia patients and found a signiifcant correlation (Cutillas-Marco *et al*., 2013). Rady and others confirmed that the FF genotype in the VDR gene is associated with a threefold risk of juvenile idiopathic arthritis (Rady *et al*., 2022). The study by Han and others shows compelling evidence that vitamin D insufficiency is linked to atherogenic dyslipidemia, and in particular, elevated small dense LDL-C levels in middle-aged adults without CVD (Han *et al*., 2021). Previous studies have linked dyslipidemia (Jorde & Grimnes, 2011) and lipid profile (Ponda *et al*., 2012) to vitamin D deficiency. Lipid levels in adulthood are also correlated with vitamin D levels, which are in turn linked to the gene's steady association with vitamin D levels in the blood (Jorde & Grimnes, 2011; Nissen *et al*., 2014; Thongthai *et al*., 2015).

Vital biological functions like cell proliferation, regulation, and differentiation, bone formation, and immune response modulation are all influenced by the endocrine vitamin D system. VDR is a member of the steroid hormone family of nuclear receptors and is responsible for the transcriptional control of a number of hormone-responsive genes by its binding to the active metabolite calcitriol. Due to the presence of VDRs in all major cardiovascular cell types, including vascular smooth muscle cells, endothelial cells, cardiomyocytes, platelets, and most immune cells, it is possible that *VDR* gene polymorphisms influence CVD. In addition, the VDR plays a crucial role in modulating the expression of several proteins that play a role in controlling the cardiovascular system. These include renin, endothelial nitric oxide synthase, and NADPH oxidase (Abouzid *et al*., 2021). The role of vitamin D and the VDR gene in Saudi Arabia is critical, as one meta-analysis study conducted in Saudi Arabia revealed that 60% of the healthy population in Saudi Arabia has a vitamin deficiency (Al-Alyani *et al*., 2018). The normal value of vitamin D deficiency in Saudi Arabia has been confirmed as 24.96 nmol/L which is below the limit (Albaik *et al*., 2016). The *VDR* gene polymorphism studies were carried out in the Saudi population with different human diseases and confirmed all forms of association (Ali *et al*., 2018; Alkhayal *et al*., 2016; Alzaim *et al*., 2022; Ansari *et al*., 2021; Mansy *et al*., 2019; Nemenqani *et al*., 2015; G. B. Saif & I. A. Khan, 2022; Taha *et al*., 2019; Zeidan *et al*., 2022). Additionally, there are limited documented studies of Saudi Arabian patients with confirmed FH. The lack of cholesterol data was one of the study limitations.

CONCLUSION

Both ApaI and BsmI polymorphisms was associated to FH via allele and genotype frequencies in the Saudi population. The Saudi Arabia VDR *gene* polymorphism link was built with FH patients.

Declarations

Conflict of interest: Not applicable for this study.

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