Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

Pharmacophore
an International Journal
ISSN 2229-5402

EVALUATION OF RELATIONSHIP BETWEEN VITAMIN D RECEPTOR GENE POLYMORPHISMS AND RHEUMATOID ARTHRITIS IN SAUDI POPULATION

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ARTICLE INFO

Received:
21th Oct 2017
Received in revised form:
20th Jan 2018
Accepted:
24th Jan 2018
Available online:
28th Feb 2018

Keywords: *Rheumatoid arthritis (RA), Vitamin D Receptor (VDR), Acetobacter pasteurianus (ApaI), Thermus aquaticus YTI (TaqI).*

ABSTRACT

This study was carried out to study polymorphisms of the Vitamin D Receptor (VDR) gene at two locations of Acetobacter pasteurianus (ApaI) and Thermus aquaticus YTI (TaqI), and the serum level of vitamin D, to find out whether there is a correlation between polymorphisms and rheumatoid arthritis. Forty female rheumatic patients and 30 healthy controls were recruited in this study. The DNA was extracted from the blood samples, then the target part of the VDR gene was isolated and amplified by Polymerase Chain Reaction (PCR), and then two enzymes were used, ApaI and TaqI, in order to digest the PCR products by Restriction Fragment Length Polymorphism (RFLP) technique. The results showed that the distribution of genotype frequency of both ApaI and TaqI showed a non-significant difference between Rheumatoid arthritis (RA) patients and the control. None of the VDR gene polymorphisms displayed an association with RA, and no significant difference was found between the patients and control groups. In conclusion, there was no significant difference between VDR gene polymorphisms and RA in our samples of Saudi women. The serum vitamin D was also showed non-significant difference between the two groups (P= 0.1992).

To Cite This Article: Sawsan O. Khoja, Rawan F. Al-Jehani, Sami M. Bahlas, Shareefa A. Algamdi, Suzan Attar, (2018), "Evaluation of Relationship Between Vitamin D Receptor Gene Polymorphisms and Rheumatoid Arthritis in Saudi Population", ***Pharmacophore, 9(1),*** *85-94.*

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting the tissues surrounding the synovial joints in hands, knees and wrists. Although it is characterized by pain, stiffness, functional impairment and swelling, it cannot be diagnosed at its onset, because its symptoms mimic other diseases. Rheumatoid arthritis occurs in middle aged individuals [1].

The onset of rheumatoid arthritis is not similar in all patients but varies according to type, number, and the pattern of joint involvement. The course of disease may be also different according to the presence or absence of several variables including genetic background, frequency of swollen joints, autoantibody in the serum and the severity of inflammatory process [2, 3]. Moreover, rheumatoid arthritis could be self-limited, non-destructive or severe destructive disease. It might be acute, sub-acute or insidious which is the most prevalent. RA might either begin as mono arthritis from one joint and then gradually spread to other joints, or as polyarthritis [4].

Before the appearance of synovitis, RA is presented in 60% of patients with fatigue, anemia, generalized weakness, anorexia and nonspecific musculoskeletal symptoms [5]. Early symptoms of RA may appear as a vague pain with gradual appearance

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without classic symptoms of joint swelling or tenderness. These unusual symptoms are usually non-specific, and may persist for a prolonged period. Early articular manifestations of RA may be indistinguishable from other rheumatic diseases. A prolonged duration of morning stiffness with arthralgia, or arthritis in a limited number of joints may be a clue for considering RA diagnosis [6].

Rheumatoid arthritis is characterized by destruction and chronic swelling of the synovial joints leading to the disability and progressive joint damage [7]. It is better to diagnose RA at its early stages in order to: prevent the development of functional limitations, to avoid surgical interventions and costly medical treatments, and to prevent damages to joints and extra-particular complications [8]. The clinically detectable onset of inflammatory arthritis is preceded by the production of auto-antibodies such as rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA). These auto-antibodies can last for years and autoimmunity is recognized by the presence of these auto-antibodies [7].

Relieving pain, preserving and improving activities of daily living, and preventing the progressive joint damage are considered the main goals of RA treatment. Nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and corticosteroids are examples of the medical treatment of the disease. Educating the patients, occupational therapy, physical therapy, orthotics, and rarely surgery are examples of the non-medical treatment of RA. It is recommended to use the DMARD therapy within three months of diagnosis of RA because it can slow or arrest improvement of the disease [9].

Vitamin D Receptor (VDR) is a 51 kDa protein classified in the family of steroid, thyroid hormone, and retinoic acid receptor genes on the basis of its similar primary amino acid structure. VDR, a member of the nuclear receptor super family, mediates the biological action of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], and regulates numerous physiological and pharmacological processes [10]. The VDR is a nuclear ligand-dependent transcription factor which in complex with hormonally active [1, 25(OH)2D3] controls the expression of various genes associated with inflammation and immune modulation. Emerging epidemiological and experimental evidence reveals that the VDR is a promising drug target in the treatment of cancers, autoimmune diseases, infections, and cardiovascular diseases, as well as bone and mineral disorders [11, 12].

Polymorphism is a genetic variant that appears in at least 1% of the population. These changes can occur in non-coding parts of the gene (introns), so they would not be seen in the protein product. Changes in these regulatory parts of the gene would then affect the degree of expression of the gene, and thus the levels of the protein. Nonetheless, changes in the exotic sequences of the DNA which do not alter the protein structure are also possible, and are called synonymous polymorphisms. Often these changes create or abolish sites for restriction enzymes to cut the DNA [13].

Polymorphisms Acetobacter pasteurianus (ApaI) and Thermus aquaticus YTI (TaqI) are located in region 3` of the VDR gene. The thymine–guanine substitution (T–G) in intron 8 resulting in polymorphism ApaI, while polymorphism TaqI, in exon 9, is caused by cytosine–thymine substitution (C–T), resulting in the codon exchange (ATC ATT), but maintaining the same amino acid isoleucine [14]. Many biological processes and diseases are affected by VDR polymorphism and this is clearly shown by association studies, the mechanism by which tile polymorphisms exert these effects is much less clear [15]. Another study reported by [16, 17] showed the positive association between VDR gene polymorphism and susceptibility to RA.

The aim of this investigation is to evaluate rheumatoid arthritis at the genetic level. Evaluation of serum vitamin D levels and genotyping of the Vitamin D receptor gene would help us determine if there is a difference between normal individuals and rheumatoid arthritis patients at the molecular level with respect to the vitamin D receptor gene.

Materials and Methods

Materials:

Venous blood samples (3ml) were obtained from the forty volunteers. Rheumatoid arthritis patients were selected from the outpatient clinic of rheumatology, King Abdulaziz University Hospital (Jeddah). Age- and sex- matched controls (n=30) with no apparent disease such as rheumatoid arthritis were randomly selected to compare with the patient data. Blood samples were obtained in two kinds of vacutainers, (1ml) of blood was obtained in ethylenediaminetetra acetic acid (EDTA) tubes which were used in the DNA extraction, and (2ml) of blood was obtained from serum separator tubes (SSTs) which were used to separate the serum for biochemical parameters.

QIAamp DNA Blood Mini Kit was obtained from QIAGEN Inc., Hilden, Germany, Cat. no. 51104. Agarose gel (Cat.No. V3125), from (Promega), Tris Acetate EDTA (TAE) from Cleaver Scientific Ltd (Batch number: 15486) and ethidium bromide (Cat. No. E1510) from SIGMA. DNA Ladder 1 kb (Catalog No. SM 0314).

Methods:

DNA extraction:

Genomic deoxyribonucleic acid (gDNA) was isolated from whole blood-EDTA using QIAamp DNA Blood Mini Kit according to [18]. The yield of DNA was eluted using elution buffer and then kept at -20°C. DNA yield and purity was determined by measuring absorbance at 260/280 nm using Thermo Scientific Nanodrop 2000 UV-Vis spectrophotometer instrument, from USA.

The polymerase chain reaction (PCR)

A bio safety cabinet was used during the preparation of the PCR reaction mixture to avoid contamination. A 2229bp fragment which includes intron8 and exon9 of the vitamin D receptor gene in chromosome 12 was amplified by PCR. PCR products were generated in a 50µl reaction mixture following instruction in GoTaq® Green Master Mix kit, catalog No. M7122. Our primers for the VDR gene were from Macrogen. The forward primer was (5′CAACCAAGACTACAAGTACCGCGTCAGTGA-3′) and the reverse primer was (5′GCAACTCCTCATGGCTGAGGTCTC-3′) [19]. The PCR reaction mixture for each sample consisted as shown in Table (1).

Table 1. The PCR reaction components.

Components	Concentration used	Quantity used
GoTaq® Green Master Mix	2X	25µl
Forward Primer (10µM)	0.2µL	1.5µl
Reverse Primer (10µM)	0.2µL	1.5µl
Template DNA	~1 µg	8µl
Deionized water	-	14µl
Total volume	50µl	50µl

The amplified PCR products were visualized using horizontal gel equipment (Model No. 329BR) and an electrophoresis power supply (Model No. 041 BR). Delete was used. The amplified products were resolved on 1% agarose gel stained with 2µl of ethidium bromide. The electrophoresis buffer used was 50X TAE “Tris Acetate EDTA”. The gel of electrophoresis was prepared using agarose gel, Tris Acetate EDTA (TAE) and ethidium bromide. DNA Ladder 1 kb was used in each run. A gel documentation system was used to visualize the PCR product.

Restriction fragment length polymorphism (RFLP).

Ten microliters of amplified PCR products were digested using (1µl) of each of the restriction enzymes which were obtained from Thermo Scientific. These enzymes are: Acetobacter pasteurianus ApaI (Cat No. FD1414) and Thermus aquaticus YTI TaqI (Cat No. FD0674). Two microlitres of 10X Fast Digest Green Buffer was added to each sample. Each sample is scaled up into 30µl using nuclease- free water (Catalog No.0581) (Table 2). Genomic polymorphisms were described as AA, TT (absence of restriction site in both alleles), aa, tt (presence of restriction site in both alleles), Aa, Tt (heterozygous) according to standard nomenclature.

Table 2. RFLP reaction components of ApaI and TaqI

Components	Concentration used		Quantity used	
	***Apa*I**	***Taq*I**	***Apa*I**	***Taq*I**
Water, nuclease-free	-	-	17µl	17µl
Buffer *Apa*I *and Taq*I	10X	10X	2µl	2µl
PCR product	~0.2 µg	~0.2 µg	10 µl (~0.2 µg)	10 µl
*Taq*I and *Apa*I	-	-	1µl	1µl
Total volume	30µl	30µl	30µl	30µl

Biochemical analysis:

Serum separator tubes (SSTs) were kept on ice for up to three hours prior to usage. Then, they were allowed to clot at room temperature for (30- 45 minutes), and centrifuged at 3000 rpm for 10 minutes, then the serum was aspirated and dispensed into 1.5ml micro centrifuge tubes and kept at -20°C until the time of assay of 25-hydroxycholecalciferol (25(OH)D3) (calcidiol), calcium, parathyroid hormone (PTH), and creatinine.

Measurement of serum vitamin D:

The Roche Diagnostics Vitamin D assay is a competitive electrochemiluminescence protein binding assay intended for the quantitative determination of 25-OH vitamin D3 in human serum and plasma. The assay employs a vitamin D binding protein (VDBP) as a capture protein, which binds to both 25-OH D3 and 25-OH D2 [20]. The assay utilizes a 3- step incubation process, which has duration of 27 minutes.

In step 1, the sample is incubated with pretreatment reagent, which releases bound 25-OH vitamin D from the VDBP. In step 2, the pretreated sample is incubated with ruthenium labeled VDBP creating a complex between the 25-OH vitamin D and the ruthenylated VDBP. The third incubation step is the addition of streptavidin-coated microparticles and 25-OH vitamin D labeled with biotin. The free sites of the ruthenium labeled VDBP become occupied, forming a complex consisting of the ruthenium labeled vitamin D binding protein and the biotinylated 25-OH vitamin D.

The entire complex becomes bound to the solid phase (by the interaction of biotin and streptavidin-coated microparticles which are captured on the surface of the electrode). Unbound substances are removed. Applying voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier.

Results are determined via an instrument specific calibration curve which is generated by 2-point calibration and a calibration master curve provided via the reagent barcode.

Vitamin D deficiency is defined as (25-OH) of ≤ 20 ng/ml (≤ 50 nmol/L). Vitamin D insufficiency is recognized as 21-29 ng/ml. The preferred level for vitamin D (25-OH) is recommended to be ≥ 30 ng/ml (≥ 75 nmol/L).

Measurement of calcium, PTH and creatinine:

Serum samples were used to measure the level of calcium (Ca) using Dimension Vista System (Cat.No. K 1023). The optimal concentration of calcium was defined as at least 2.2-2.62 mmol/L. Parathyroid hormone (PTH) was measured using ADVIA Centaur and ADVIA Centaur XP system Cat.No.100 Test kit 04385983 (129501) or 500 Test kit 06587575 (129502). The optimal concentration of PTH was defined as 1.60-6.90 pmol/L. Creatinine was measured by using Dimension Vista System (Cat.No.1033A). The optimal concentration was defined as 60-100 µmol/L.

Statistical analysis:

Mean±SD (standard deviation) values were calculated in all investigated parameters by using MegStat® version 9.0 software. Genotype frequencies of the VDR gene polymorphisms in the patient and control groups were determined according to Hardy–Weinberg equilibrium by using the chi-square analysis (2×2 contingency tables) and Fisher's exact probability test. The frequencies of different polymorphisms among RA patients and the controls were calculated. Association between clinical laboratory markers and SNPs screened were determined by one-way analysis of variance (ANOVA). P values less than 0.05 were considered significant. Rare genotypes (n=0 in any group) were excluded from the analysis. Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles.

Results and Discussion

Analysis of ApaI and TaqI polymorphisms in the VDR gene.

Diagram (1) showed that the PCR product of VDR gene which is ~ 2229 bp. Also shows the digestion products of TaqI enzyme.

DNA fragments were resolved on 1% agarose gel. Genotypes were determined as follows; AA (2229), Aa (2229-1700-529 bp) or aa (1700-529 bp) for ApaI polymorphism as shown in Diagram (2); TT (1982-247 bp), Tt (1982-1780-247-202 bp) or tt (1780-247-202 bp) for TaqI as shown in Diagram (3).

The distribution of genotypes and results of the ApaI and TaqI restriction enzymes of the patients and controls are shown in Tables (3 and 4). The allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. None of the studied samples showed Tt polymorphism.

There have been several studies on the possible association of RA, clinical manifestations of RA, and VDR polymorphisms. The results have been controversial due to ethnicities and geographic variations [21, 22].

VD deficiency was commonly associated with RA severity and/or exacerbation. The VDR gene, which is considered as a pleiotropic gene, is associated with multiple inflammatory and allergic diseases. VDR gene polymorphisms may affect VD structure and function, as well as the VD serum levels. Genetic association studies on VDR polymorphisms and RA disease are conflictive, and the role of VDR polymorphisms remains unclear. The reasons for this disparity may be the small sample sizes, low statistical power, differences in ethnicities, extensive geographic variations, interactions with other genetic or environmental factors and clinical heterogeneity [23]. The importance of VDR polymorphism study in population arises from the differences between genotypes and alleles according to the ethnicity. This requires comparison of genotypes and allele frequency between healthy individuals and patients in each population, and then comparing the genotype and allele frequency with other different populations [24].

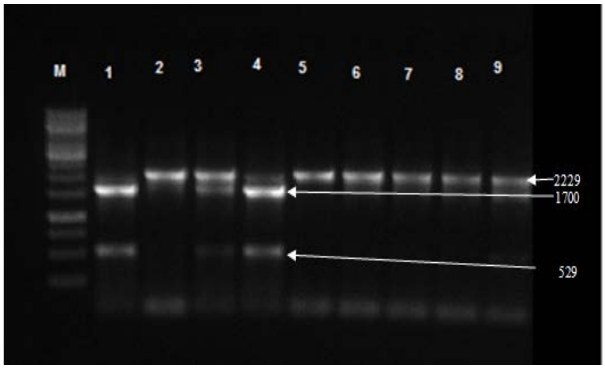

Diagram 1. Photograph of 1%agarose gel showing results of PCR product, -ve control and TaqI estriction enzymes. **Lane M:** 1kb DNA marker. Lane 2: negative control, lane. **3: PCR** product. Lanes 4 and 5: TaqI restriction enzyme

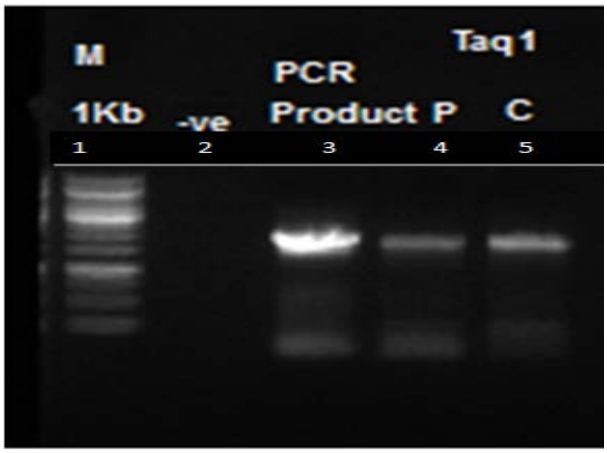

Diagram 2. Photograph of 1% agarose gel showing results of ApaI restriction enzyme. Lane M: 1 kb DNA Ladder, Lane 1, aa genotype (1700 bp and 529 bp, wild), Lanes 2, 5, 6, 7: AA genotype (2229bp, homozygous dominant), and lanes 3, 4, 8, 9: Aa genotype (2229 bp, 1700 bp and 529 bp, heterozygous).

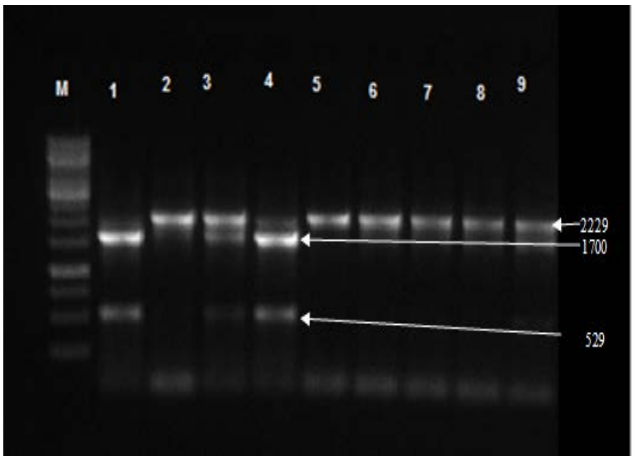

Diagram 3. Photograph of 1% agarose gel showing the results of TaqI restriction enzyme. Lane M: 1 kb DNA marker, Lanes 1, 2, 3, 4, 5: tt genotype (1780 bp, 247 bp and 202 bp, wild)

Table 3. Summary of the frequency and percentage of the alleles and the genotypes of the entire RA samples

VDR polymorphisms	Alleles	NO. of samples	(100%)
ApaI Alleles	A	25	61.25
	a	15	38.75
	Total	40	100
TaqI Alleles	T	22	55
	t	18	45
	Total	40	100
ApaI Genotypes	AA	10	25
	Aa	29	72.5
	aa	1	2.5
	Total	40	100
TaqI Genotypes	TT	22	55
	Tt	0	0
	tt	18	45
	Total	40	100

Table 4. Summary of the frequency and percentage of the alleles and the genotypes of the entire control samples

VDR polymorphisms	Alleles	NO. of samples	(100%)
ApaI Alleles	A	18	60
	a	12	40
	Total	30	100
TaqI Alleles	T	0	0
	t	30	100
	Total	30	100
ApaI Genotypes	AA	12	40
	Aa	12	40
	aa	6	20
	Total	30	100
TaqI Genotypes	TT	0	0
	Tt	0	0
	tt	30	100
	Total	30	100

Characteristics of the subjects according to their ApaI and TaqI genotypes

The studies of samples' biochemical analysis with respect to ApaI and TaqI are represented in Tables (5, 6 and 7). All samples of control group had tt genotype; therefore, we could not compare the results for the TaqI enzyme for the control group. In the two polymorphisms, there were no significant difference (P >0.05) in total vitamin D, calcium, PTH values between different genotypes. Creatinine showed non-significant difference in the ApaI and TaqI enzymes in the patients group, but showed significant difference in the ApaI enzyme in the control group. Due to these reasons we conducted our study on a sample of Saudi population to find the relationship between VDR gene polymorphisms ApaI and TaqI, and vitamin D levels and occurrence of RA.

We also measured biochemical parameters such as PTH, calcium and creatinine. We divided our population into two groups: patients and controls. Results showed non-significant difference of vitamin D between patients and controls (P= 0.1992). The result of PTH, calcium and creatinine also showed non-significant difference between patients and controls (P= 0.7621, P= 0.0585, P= 0.1554, respectively).

Our analyses indicated that ApaI and TaqI VDR polymorphisms were non-significantly associated with RA risk. Effectively, TaqI, as a synonomous polymorphism, does not change the amino acid sequence of the encoded protein, but is involved in regulating the stability of VDR mRNA and/or protein translation efficiency [25]. The ApaI and TaqI polymorphisms are located in intron 8 and exon 9 at the 3' untranslated region (3'UTR) of the VDR gene, respectively. This region is a part of the ligand-binding domain of the VDR and therefore, any structural variation in the protein might lead to a differential binding specificity of vitamin D [26]. Although the functional effects of the ApaI and TaqI SNPs remain unknown, they have been reported to be associated with an increased susceptibility to several autoimmune diseases including diabetes [27, 28], multiple sclerosis [29], Graves 'disease [30] and asthma [31].

Table 5. Characteristics of the 40 rheumatoid patients according to their ApaI genotypes distribution

Variables	Genotypes			ANOVA P-value
	Aa n=(1)	Aa n=(29)	AA n=(10)	
25(OH)D_3 (nmol/L)	47.26±17.40	53.28±25.17	55.51±0	0.7756
Calcium (mmol/L)	2.073±0.14	2.18±0.198	2.09±0	0.3220
PTH (pmol/L)	2.95±2.01	4.81±7.47	1.41±0	0.6784
Creatinine (umol/L)	31.93±31.32	61.97±29.49	25.40±0	0.0228*

Variables are presented as means ± SD *Significant differences

Table 6. Characteristics of the 30 healthy controls according to their ApaI genotypes distribution

Variables	Genotypes			ANOVA P-value
	Aa n=(12)	Aa n=(12)	AA n=(6)	
25(OH)D_3 (nmol/L)	44.67±28.42	46.72±25.23	38.43±24.54	0.8206
Calcium (mmol/L)	2.28±0.12	2.26±0.11	2.09±0.09	0.0100*
PTH (pmol/L)	3.89±1.65	3.87±1.58	3.92±2.44	0.9989
Creatinine (umol/L)	59.75±11.02	55.86±6.96	40.57±8.89	0.0010*

Variables are presented as means ± SD *Significant differences

Table 7. Characteristics of the 40 rheumatoid patients according to their TaqI genotypes distribution

Variables	Genotypes			ANOVA P-value
	Tt n=(18)	Tt n=(0)	TT n=(22)	
25(OH)D_3 (nmol/L)	58.17±25.52	--	44.08±17.34	0.0534
Calcium (mmol/L)	2.16±0.21	--	2.15±0.16	0.8425
PTH (pmol/L)	3.54±2.30	--	5.13±9.38	0.4465
Creatinine (umol/L)	68.75±39.03	--	55.34±10.49	0.1658

Variables are presented as means ± SD

The frequencies (%) of alleles and genotypes of the ApaI and TaqI loci in rheumatoid arthritis patients and controls are presented in (Tables 4.8 and 4.9). The allelic frequencies of 'A' vs 'a' and 'T' vs 't' in the patient group were 61.25 vs 38.75% and 55 vs 45%, respectively, whereas, the allelic frequencies of 'A' vs 'a' and 'T' vs 't' in the control group were 60 vs 40% and 0 vs 100%, respectively. The frequencies of AA, Aa and aa genotypes of VDR gene ApaI polymorphism in the patients were 25, 72.5 and 2.5% and in the controls were 40, 40 and 20%, respectively. The frequencies of TT, Tt, and tt genotypes of VDR gene TaqI polymorphism in the patients were 55, 0 and 45% and in the controls were 0, 0 and 100%, respectively. None of the studied subjects had Tt genotypes.

Hardy–Weinberg equilibrium (HWE) calculations that were made for the ApaI showed no significant difference between observed values and the expected values, both groups were out of HWE ($P < 0.05$). Whereas, the TaqI polymorphism in the patient group was out of HWE ($P < 0.05$), but TaqI polymorphism in the control group was within HWE ($P > 0.05$). The 2 % of ApaI polymorphism for RA was 25.07 and 47 for controls, whereas, the 2% of TaqI for RA was 81.14, and 0.03 for controls.

In the present study, there was non-significant difference in the distribution of the heterozygous mutant vitamin D receptor ApaI (Aa) genotype in RA patients and controls with odds ratio (OR) = 0.34 (95% CI= 0.12-1.01(, and risk ratio (RR) = 0.64(0.39-1.06) with a P-value=0.06. The distribution of homozygous mutant (aa) also showed a non-significant difference with OR= 5 (95% CI= 0.51-48.75), and risk ratio= 3.2 (0.49-20.67) with a P-value= 0.2.

The TaqI enzyme showed non-significant effect. The heterozygous mutant (Tt) was not found either in the patients or controls, therefore, the OR and RR cannot be calculated. The homozygous mutant (tt) also showed non-significant effect. The OR=NA (95% CI=NA), and risk ratio=3(1.85-3.84) with a P-value>0.05.

Previous studies showed that rs731236(Taq1) TT polymorphism have been associated with accelerated generalized bone loss among RA, and associated with increased risk of Hashimoto's thyroiditis among Turkish patients [32]. Cutolo and colleagues and Haque and Bartlett found an inverse relationship between vitamin D levels and disease activity in RA [33, 34]. By contrast, others did not find a relationship between vitamin D deficiency and disease activity in RA. In the study by Braun-Moscovici and colleagues, they found no correlation between vitamin D levels and disease activity among 85 patients with RA [35].

Genotypes frequencies for wild, heterozygous and homozygous variants of ApaI polymorphic allele among patients group were 25, 72.5 and 2.5, respectively, and among control group were 40, 40 and 20, respectively, with non-significant associations ($P = 0.06$) between AA and Aa genotypes and ($P= 0.2$) between AA and aa genotypes. On the other hand, the genotypes' frequencies for wild, heterozygous and homozygous variants of TaqI polymorphic allele in patients were 55, 0 and 45, respectively, and among control group, they were 0, 0 and 100, respectively, with insignificant associations ($P=1$) between TT and Tt genotypes, and ($P \geq 0.05$) between TT and tt genotypes.

Table 8. Genotypes and allele frequencies of VDR gene ApaI enzyme for patients and controls

VDR polymorphism	Frequencies%	P value1		Risk Ratio	Odds Ratio
	Control (n=30)	Patients (n=40)		(95%CI)	(95%CI)
Genotypes					
AA	25% (n=10)	40% (n=12)		1.0 (Reference)	1.0 Reference
Aa	72.5% (n=29)	40% (n=12)	0.06	0.34 (0.12-1.01)	0.64 (0.39-1.06)
aa	2.5% (n=1)	20% (n=6)	0.2	5 (0.51-48.75)	3.2 (0.49-20.67)
Alleles					
A	61.25% (n=25)	60.0% (n=18)		1.0 (Reference)	1.0 Reference
a	38.75% (n=15)	40.0% (n=12)	0.88	1.05 (0.60-1.87)	1.03 (0.77-1.37)

[1]Fischer exact probability test (two- sided chi-square probability)

Table 9. Genotypes and allele frequencies of VDR gene TaqI enzyme for patients and controls

VDR polymorphism	Frequencies%		P value1	Risk Ratio (95%CI)	Odds Ratio (95%CI)
	Control (n=30)	Patients (n=40)			
Genotypes					
TT	55% (n=22)	0% (n=0)		1.0 (Reference)	1.0 Reference
Tt	0% (n=0)	0% (n=0)	1*	NA	NA
tt	45% (n=18)	100% (n=30)	>0.05*	NA	3 (1.85-3.84)
Alleles					
T	55% (n=22)	0% (n=0)		1.0 (Reference)	1.0 Reference
t	45% (n=18)	100% (n=30)	>0.05*	NA	3.22 (2.53-4.11)

[1]Fischer exact probability test (two- sided chi-square probability)

The extent of vitamin D deficiency/ insufficiency in rheumatoid arthritis patients

We tried to correlate the levels of vitamin D_3 with Apa1 and Taq1 genotypes. 25-hydroxyvitamin D3 levels were analyzed for 40 rheumatic patients, who were segregated into low vitamin D (≤50 nmol/L), those who were insufficient (51-74 nmol/L) and finally in the recommended level (≥75 nmol/L). For the Apa1 polymorphism, we observed that 50% of the AA were vitamin D deficient, and 50% were insufficient. For the Aa, 48% were deficient, 28% were insufficient and 24% were in the recommended level. For the aa genotype, 100% were insufficient for the vitamin D. On the other hand, for the Taq1 enzyme, we observed that 36.4% of TT genotype were deficient, 36.4% were insufficient, and 27.2% were in the recommended level. For the tt genotype, 61.11% were deficient, 33.33% were insufficient, and the rest 5.56% were found to be in the recommended level. The data is shown in Table (10).

Vitamin D (VD) is a potent regulator of calcium homeostasis [36, 37]. In the study of [38], in 29 patients with RA, it had decreased serum calcium concentrations. In the present study, calcium levels were found to be low in 26 out of the 40 patients with RA.

The extent of vitamin D deficiency/ insufficiency in control

For the Apa1 polymorphism in the control group, we observed that 75% of AA was vitamin D deficient, 8.33% were insufficient, and the rest 16.67% were in the recommended level. For the Aa, 50% were deficient for vitamin D, 33.33% were insufficient for vitamin D, and 16.67% of Aa genotype were in the recommended level of vitamin D. For aa polymorphism, 50% were deficient for vitamin D, and 50% were insufficient for the vitamin D. Whereas, for the Taq1 enzyme of the control group was homozygous mutant, when the genotype was tt. 60% were found to be deficient for vitamin D, 23.33% were insufficient, and the rest 16.67% were found to be in the recommended level of vitamin D. The data is shown in Table (11).

Table 10. Correlation of Apa1 and Taq1 genotypes with the vitamin D3 levels in rheumatoid arthritis patients

Genotypes	Deficient ≤50 nmol/L	Insufficient 51-74nmol/L	Recommended ≥75nmol.L	n= (40)
AA	50 (5)	50 (5)	0 (0)	10
Aa	48 (14)	28 (8)	24 (7)	29
aa	0 (0)	100 (1)	0 (0)	1
TT	36.40 (8)	36.40 (8)	27.2 (6)	22
Tt	0 (0)	0 (0)	0 (0)	0
tt	61.11 (11)	33.33 (6)	5.56 (1)	18

Table 11. Correlation of Apa1 and Taq1 genotypes with the vitamin D3 levels in controls

Genotypes	Deficient ≤50 nmol/L	Insufficient 51-74nmol/L	Recommended ≥75nmol.L	n= (30)
AA	75 (9)	8.33 (1)	16.67 (2)	12
Aa	50 (6)	33.33 (4)	16.67 (2)	12
aa	50 (3)	50 (3)	0 (0)	6
TT	0 (0)	0 (0)	0 (0)	0
Tt	0 (0)	0 (0)	0 (0)	0
tt	60 (18)	23.33 (7)	16.67 (5)	30

Conclusion

This case-control study did not find a significant difference in genotype and allele frequency at positions ApaI and TaqI of the VDR gene between RA patients and the control group. The data nullified any significant association of VDR gene polymorphisms with RA in our study group. Polymorphic marker alleles did not have an effect on vitamin D levels of Saudi rheumatic patients. We also found no statistical difference between the serum levels of vitamin D of the rheumatoid arthritis and healthy controls. Further merit investigation will be required to elucidate these findings using larger sample size.

Acknowledgements

The authors acknowledge King Abdulaziz City for Science and Technology (KACST) for technical and financial support.

Declerations

Conflict of interest: The authors declare no conflict of interest associated with this work.

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