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VDR, CYP24A1 and KL Gene Variations Significantly Associated with CKD Pathogenesis: A Comparative Genetic Study

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KEYWORDS Chronic Kidney Disease. Single Nucleotide Polymorphism. Amplification-Refractory Mutation System-Polymerase Chain Reaction

ABSTRACT Allelic variation and genomic instability of the VDR, KL and CYP24A1 variations are influencing the pathogenesis of chronic kidney disease (CKD) in the computational analysis. To correlate this observation this study aimed to screen the population frequencies of the following alleles in the Indian populations. The *VDR gene*: rs121909800, rs121909797; *CYP24A1 gene:* rs387907322, rs114368325, and *KL gene:* rs9536314, rs649964 in CKD patient's genotyping was done from blood sample DNA using the ARMS-PCR method and significant results were statistically validated through MDR analysis. In this study, 120 CKD cases and 120 control subjects were included. The allele distribution and genotype of the rs121909800 variant were significantly associated with CKD patients (Variant Allele Frequency or VAF = 11.67) (*p-value*: 0.03), versus control subjects (VAF=4.17), respectively. The *CYP24A1* polymorphic variants rs387907322 and rs114368325 are significantly associated with CKD patients. The disease association was confirmed through Multifactor Dimensionality Reduction (MDR) analysis. Hence, the researchers concluded that the associated genes with specific variants may be considered a potential marker for the early diagnosis of CKD.

INTRODUCTION

The pathophysiology of CKD is influenced by a combination of environmental and genetic factors. In addition to several systemic effects, it is associated with a profound immunobiological, metabolic, and hydroelectrolytic imbalance (Cañadas-Garre et al. 2019). Gene polymorphisms have been shown to exert an influence on CKD susceptibility and pathogenesis (Corredor et al. 2020). Several epidemiological studies have investigated the relationship between VDR, CYP24A1, and KL gene polymorphisms and the risk of CKD (Solache-Berrocal et al. 2021). Renal function and bone mineral density have been shown to be inversely related in several studies (Li et al. 2019).

Vitamin D is a prohormone that is converted into Vitamin D_2 and Vitamin D_3 by the liver and kidneys. Activated Vitamin D_3 plays a vital role in calcium and phosphate homeostasis through its ability to absorb through the intestinal, mobilise calcium formation in bones, regulate cell differentiation, and promote renal excretion (Bikle 2021). Numerous gene polymorphisms are involved in the pathogenesis of CKD (Corredor et al. 2020). In particular, the VDR, CYP24A1, and Klotho (KL) genes polymorphisms play an important pathogenetic role in the cause of CKD (Solache-Berrocal et al. 2021). These genes are mainly responsible for circulating levels of Vitamin D, leading to CKD also SNPs of these polymorphisms associated with CKD pathogenesis it was reported in several populations.

A VDR gene has eight codon exons (2-9), as well as six untranslated exons (1a-1f), and is situated on chromosome 12q12.14 (Imani et al. 2020). Many SNPs occur often in the VDR gene's introns or 3' untranslated region (UTR). Changes in this region influence the mRNA stability as well as protein translation efficacy. Transcriptional regulation, which is ultimate, affects the VDR protein levels (Kongsbak et al. 2013). In humans, the VDR gene was reported with more than 14 different polymorphisms, which might play an influential role in the binding affinity of the calcitriol to VDR. ApaI (rs121909800), BsmI (rs1544410), FokI (rs2228570), and TaqI (rs121909797) are the four most often studied Single Nucleotide Polymorphisms (SNPs) (Hoseinkhani et al. 2021) as well as reported deleterious SNPs by computational studies (Jayaraj et al. 2021). CYP24A1 gene is located in chromosome 20q13.2-q13. The mutation or SNP in the CYP24A1

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enzyme also alters the Vitamin D action through metabolising Vitamin D_2 and Vitamin D_3. Two SNPs are widely studied namely, rs387907322 and rs114368325, and have a pathogenic role and are involved in Vitamin D dysregulation related deficiencies (Hill et al. 2016). KL gene structure contains five exons in the coding region, which is located in chromosome 13. Recent clinical findings (Donate-Correa et al. 2016; Yokoyama et al. 2019), as well as computational studies (Jayaraj et al. 2021) suggested that, there are ten or more SNPs or mutations of the KL gene, which has a pathogenic effect to cause CKD, and is associated with mineral bone density, and cardiovascular disease. Three mutations of the KL gene are more vulnerable such as F352V, C370S (substitution of amino acids), and K385K (silent mutation), which were present in exon 2. These mutations influence KL activity and metabolism and also affect human cognition and intelligence (Hanson et al. 2021). Many pathological and clinical conditions are linked to these polymorphisms, including vitamin D deficiency, osteoporosis, and breast cancer (Laitman et al. 2012; Fuhrman et al. 2013; Zhang et al. 2018).Patients with CKD may also have a chance for secondary hyperparathyroidism (sHPT) due to the abnormal calcium metabolism (Hyderand Sprague 2020). In secondary hyperparathyroidism, there is an excess of parathyroid hormone that accelerates bone turnover. Calcium levels are induced by bone resorption. Increased calcium levels inhibit Vitamin D_3 and intact PTH (iPTH) synthesis (Drüeke 2021). Thus, studying the VDR, CYP24A1, and KL genetic variants may be helpful in assessing Vitamin D and bone metabolism.

Objectives

The researchers aimed to determine whether VDR, CYP24A1, and KL gene polymorphisms influence the South Indian population. To compare and correlate the computational finding of above, the genetic variations were selected for the study, following deleterious alleles were analysed for the finding gene and genotype frequencies of respective alleles. The researchers selected CKD patients between the age groups of 30 and 70 years, matched for age and sex, to confirm the correlation effects of VDR, CYP24A1, and KL gene polymorphisms. The genotypes and haplotype associations for the above mentioned alleles of VDR, CYP24A1, and KL genes were determined through the Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method for this study population. To determine the significance of the study population, statistical validations were used.

MATERIAL AND METHODS

Blood Sampling and Study Population

The 120 CKD patients and 120 control subjects were studied. This study was approved by the Alagappa University Institutional Ethical Committee (IEC/AU/2016/1/15). Data were collected from all the participated patients and control subjects after obtaining an informed consent form (Annexure-I-III).Age distribution of patients as well as control subjects range from 30 to 70 years old. Five millilitres of peripheral blood samples were collected from all the participants after the examination of the nephrologists. The majority of the patients included for this study belonged to the category stage II to IV of CKD. Age and sex matched healthy volunteer control subjects (n=120) were selected with normal blood pressure, and free from the renal failure family and history of CKD. All participants filled out a questionnaire containing their clinical details and their family history. The participants were all from the south Indian state of Tamil Nadu who were Dravidian ancestors. The researchers collected blood samples from patients at Lee Kidney Care, Madurai. Moreover, control samples were collected from the same location and origin as the samples collected from the volunteers. Centrifugation at 3000 rpm for 10 minutes separated blood cells from plasma and serum, and tubes were labelled and kept at -80°C until analysis. A sample of EDTA blood is used to isolate DNA for molecular studies by Miller's method (Karthikeyan et al. 2013; Singh et al. 2015). The researchers quantified all samples with a Nanodrop spectrometer (Thermo Scientific, USA) at OD260/OD280 ratio. Genotyping was conducted by the coupled ARMS-PCR method.

Primer Designing ARMS-Polymerase Chain Reaction

Primers were designed for genes associated with CKD, such as VDR, CYP24A1 and KL, and have been designed, which are six SNPs (rs387907322,

rs114368325, rs121909800, rs121909797, rs9536314, and rs649964). The previous computational analyses revealed these polymorphisms might have a deleterious role on the CKD and these polymorphic sites were analysed to identify the SNPs in the site. For this study, gene sequences were obtained/downloaded from the National Centre for Biotechnology Information's database (www.ncbi.nlm.nih.gov). The primer BLAST program is used for PCR primer design, to construct primers to the target sequence template. An analysis of the potential product's specificity to the targets was performed using BLAST against the user-specified databases. Integrated DNA technologies, USA supplied all primers used in this study. It was shown in Table 1 with specific information. Primers were selected/designed based on Primer 3 software. For all the SNPs, two sets of primers were designed. One set is an outer primer [named 1,4], and it covers the deleterious SNPs, which is present in the middle of the amplicon. Another set is an SNP specific at the 3' site of the forward and reverse primers respectively [2 and 3]. For each amplification three primers should be added [1,4,2/3] to amplify wild type/mutant allele respectively (Fig.1).

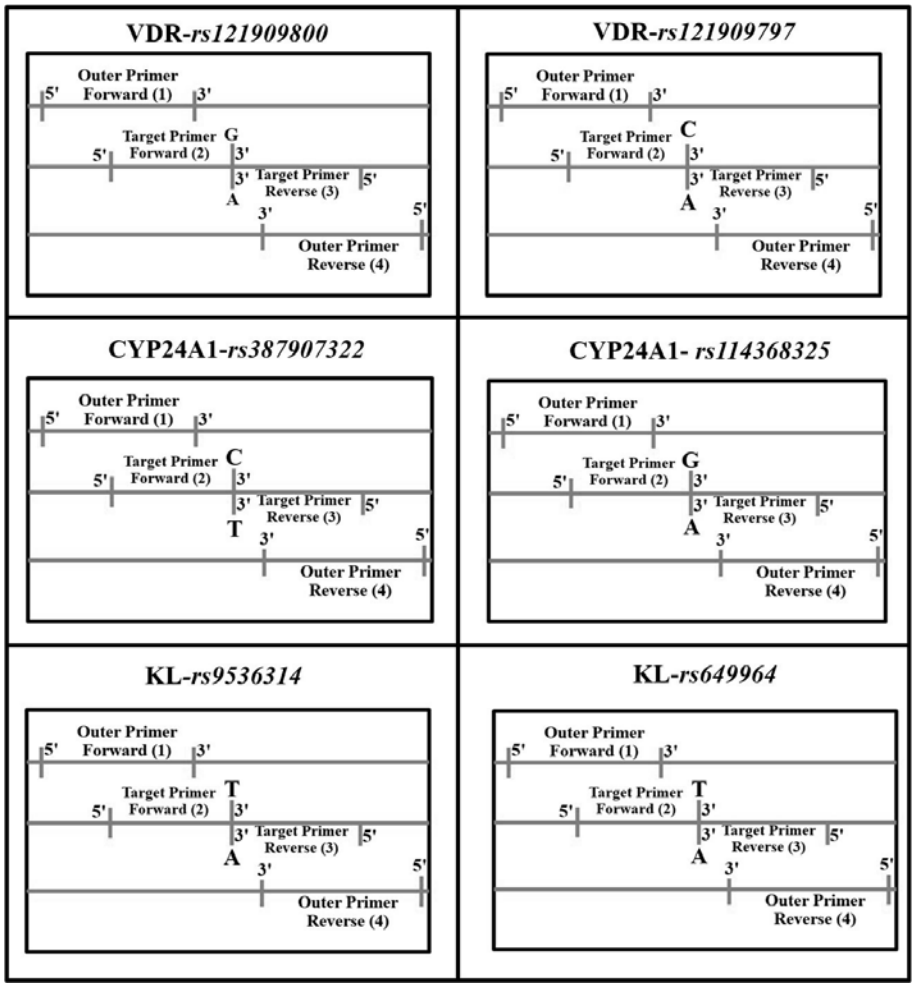

Fig. 1.Schematic representation ofARMS-PCR analysis of VDR, CYP24A1 and KL gene polymorphisms

Table 1: List of outer and target primers given in the table with the respective amplicon size

	VDR-rs121909800 polymorphism (G'!A) *Outer primers*	*Product size*	*Targeted gentotype/ allele*	*Band pattern*
(1) 5' TGG CCT GCT TGC TGT TCT TA 3'	(4) 5' TTC CAT GGA CAT TGT AAG GA 3'	285[1 to 4]	Control	**GG AA**
Inner Primers Wild	2) 5' TGT GGA GAC CGA GCC ACT GG 3'	142[2 to 4]	GG	
Mutant	3) 5' CAT AGC ATT GAA GTG AAA GT 3'	182[1to 3]	AA	
	VDR-rs121909797 polymorphism (C→A) *Outer primers*	*Product size*	*Targeted gentotype/ allele*	*Band pattern*
1) 5' CCT GCC TGA CCC TGG AGA CT 3'	4) 5' ACC AAG ACC CTC CTG CTC CT 3'	274[1 to 4]	Control	**CCAA**
Inner Primers Wild	2) 5' CCT GTG AAG GCT GCA AAG GC 3'	182[2 to 4]	CC	
Mutant	3) 5' GGA GGG CTC ACC TGA AGA AT 3'	131[1to 3]	AA	
	KL- rs9536314 polymorphism (T→A)			
1) 5' TAC TTC TTT CCG TCC CAC TC 3'	4) 5' TGG TTA AAT TCA AGG TCA AT 3'	372[1 to 4]	Control	**TTAA**
Inner Primers Wild	2) 5' TTC ATC TAT TCT GCC TGA TT 3'	186[2 to 4]	T T	
Mutant	3) 5' TTT TTC TCA GAT TCA GTA AT 3'	225[1to 3]	AA	
	KL- rs649964 polymorphism (T→A)			
1) 5' AGA AAT GAC CGA CAT CAC GT 3'	4) 5' TGT GTG TTG ATG TTG GAGAT 3'	295[1 to 4]	Control	**TTAA**
Inner Primers Wild	2) 5' CCA TGT ACA TAA TAT CCA AT 3'	188[2 to 4]	T T	
Mutant	3) 5' GCA GCC CGT CAT CGA TTC CT 3'	146[1to 3]	AA	
	CYP24A1–rs387907322 polymorphism (C'!A)			
1) 5'GTT CCT GAA GTT GCA GCT GG3'	4) 5' AAT CAC CCG AAT TGC ATT CT 3'	290[1 to 4]	Control	**CCAA**
Inner Primers Wild	2) 5' GAA GAC TGG CAG CGG GTC CC 3'	198[2 to 4]	CC	
Mutant	3) 5' GTT TCT TTT GAA GGC ACT CA 3'	131[1to 3]	AA	
	CYP24A1- rs114368325 polymorphism (G→A)			
1) 5' TTT AAC TAA CTC AAG AAG 3'	4) 5' ACC ATC TCT GCA TTC CC 3'	406[1 to 4]	Control	**GGAA**
Inner Primers Wild	2) 5' GAG TGT ACC ATT TAC AAC TG 3'	192[2 to 4]	GG	
Mutant	3) 5' GTT GCC TTG TCA AGA GTC CT 3'	253[1to 3]	AA	

(1): Outer Forward Primer, (2) Target Primer for wild type (Forward), (3) Target Primer for mutant allele (Reverse), (4) Outer Reverse Primer

Primer 1 is a forward outer primer.

Primer 4 is a reverse outer primer.

Primer 3 is a forward of the inner primer (3' carry the wild type of the allele).

Primer 2 is a reverse of inner primer (it carries the mutant allele of SNPs at the 3' site).

Based on the SNP present/absent bands will be amplified, wherein one is common (1,4 outer primer) to another band from primer 2 to outer primer (2_{nd} band).

Similarly to confirm another type of genotype (mutant) in the second primer combination (1,4, and 3) were added and amplified according to the condition set in the PCR. Primer 1,4 amplifies the outer sites of SNPs (1,3) (inner) and amplifies the wild type of genotype PCR product (if this particular genotype is present). If the inner amplified band indicates the genotype of the wild type is present. Whereas (2,4) amplified indicates that mutant allele is present at the site.

Alternatively, if both sets are amplified it indicates that (1,3; 2,4) is heterozygous.

Genotypes were classified accordingly based on the amplicons as follows:

1. Lane 1 only amplified (2 bands) is homozygous mutant (1,4; 1,2)

2. Lane 2 alone amplified (2 bands) is homozygous wild (1,4; 1,3)

3. Both lanes 1 and 2 amplified is heterozygous genotype. All the ARMS-PCR reaction was standardised for stringent annealing temperature through the Eppendorf gradient PCR machine. Later all the other patients and control were amplified according to the standardised TA.

Polymerase Chain Reaction (PCR) was used to amplify the isolated genomic DNA. *In vitro* amplification of specific DNA sequences using sequence-specific synthetic oligonucleotides is undertaken by the PCR method, which is considered to be very sensitive and powerful. An oligonucleotide primer pair is the base of PCR, which is designed to act as an important component for forward or sense primers, which synthesise DNA towards antisense primers and vice versa. The *Taq polymerase* (a heat-stable polymerase) catalyses the synthesis of a complementary DNA strand to a template DNA from both the 5' and 3' directions, resulting in the generation of a DNA region flanked by two primers in the PCR. Nucleic acid sequences can be amplified at very low concentration levels in complex mixtures. The resulting amplification is rapid and unlimited. ARMS-PCR was the characterization of SNPs of respective selected genes. Specifically, the inner primers were used as a control to check if the DNA was amplified intact, while the outer primers targeted DNA fragments with different lengths, which are essential to compare and analyse the two alleles. The required PCR product was amplified using the ARMS-PCR method. PCR condition, size, and score information were listed in Table 2 and schematic illustration of the amplification is also shown in Figure 1.

Agarose Gel Electrophoresis

The amplified ARMS-PCR products were electrophoresed in 2 percent of agarose gel for 2 hours at 50 V, along with molecular weight markers. The agarose was weighed and 100 ml of a 1X TBE (Tris Boric Acid-EDTA) buffer was added, which was dissolved by heating in a microwave until a clear transparent solution was obtained. It was cooled until it reached a warm temperature (60^{o}C), after which 0.1 µl of EtBr (0.5 mg/ml) was added and mixed very well. The gel was prepared and run in a horizontal electrophoresis chamber with 0.5X TBE as a running buffer with 50-100 V for 2 hours. The amplified products were visualised using Ebox-VILBER.

Statistical Analysis

The frequencies of VDR, CYP24A1, and KL genotypes in case and control participants were examined using the Chi-square test. The researchers used the odds ratio (OR) and 95 percent confidence interval (CI) to assess the significance of a link between genotype frequencies and disease intensity. A p-value of 0.05 was used to indicate statistical significance in this investigation. The Hardy-Weinberg Equilibrium (HWE) was assessed to find out which marker allele frequencies diverged from the HWE deviation. All statistical computations were performed using SPSS (IBM Corp. released IBM SPSS Statistics for Windows, Version 20.0). The power of the study was estimated by calculating PS-Power and the sample size.

MDR Analysis

Multifactor Dimensionality Reduction (MDR) analyses combine multiple-locus genotypes and environmental factors to identify risky alleles associated with disease.

Table 2: SNP ID, Annealing temperature and genotype count for VDR, CYP24A1, and KL polymorphisms for patients and control subjects

SNP ID	*Annealing Temperature (TA °C)*	*W/H/M*	*Genotype count*	
			Patients	*Controls*
rs387907322	60	W-CC	82	77
		H-CT	30	38
		M-TT	8	5
rs114368325	60	W-GG	74	81
		H-GA	34	31
		M-AA	12	8
rs9536314	54	W-TT	78	74
		H-TA	31	40
		M-AA	11	6
rs649964	60	W-TT	67	74
		H-TA	40	38
		M-AA	13	8
rs121909800	54	W-GG	72	70
		H-GA	34	45
		M-AA	14	5
rs121909797	60	W-CC	73	76
		H-CA	31	32
		M-AA	16	12

A critical aspect of the MDR technique is categorising genotype combinations into categories based on a new variable. Gene-gene interaction or epistasis can be determined, characterised, and interpreted with this induction method. An MDR approach alters the space of data representation by producing a new variable from two or more variables, making it easier to identify interactions among variables (Singh et al.2015). Based on simple binary categorisation, each genotype combination was classified as high-risk or low-risk in the original MDR. MDR classification should be improved by using odds ratios (OR). In the MDR, the OR is used to quantify the risk of a given genotype combination. The OR is calculated by dividing the genotype frequency ratio between a case and control group by the genotype frequency ratio between all sample groups. Based on the frequency table, the researchers predicted both genetic synergistic effects (epistasis) and associations between locus and genes. An illustration of the findings was constructed using a dendrogram and circle graph based on the prediction.

RESULTS

Studying the correlation between genetic polymorphisms and disease association is a hallmark for dissecting out the molecular mechanisms of complex diseases. The researchers understand that the molecular regulatory mechanism of CKD has examined VDR, CYP24A1 and KL polymorphisms in the South Indian population. The results of PCR amplicons were segregated based on molecular size and the presence and absence of the alleles was detected electrophoresis in 0.7 percent of Agarose gel followed by stained with Ethidium Bromide and visualised using UV transilluminator or Gel documentation system (Ebox-VILBER) shown in (Figure 1). Based on inclusion and exclusion criteria recommended by the international CKD association and World Health Organisation norms, 120 patients were included for this study. A similar number of age and sex matched healthy volunteer control subjects were included in this study. Among the included subjects, patients and controls were distributed respectively with 76.67 percent male (n=92) and 23.33 percent female (n=28) patients, and 70.83 percent males (n=85) and 29.16 percent females (n=35) in control subjects. The mean age of the male and female patients are 53.9 ±12.3 and 54.40 ± 10.42 years, respectively. Whereas in control subjects male and female patientsare 43.71 ± 14.17and 43.90 ± 13.57 years, respectively (Table 3).

Distribution of Genotypes

A genotype distribution analysis between the six studied polymorphisms (rs387907322, rs114368325, rs9536314, rs649964, rs121909790, and

Table 3: Demographic distribution of the CKD patients and Control subjects

	Cases (n=120)			*Controls (n=120)*		
	Male (%) ±SD	*Female (%)*	*Total (%)*	*Male (%) ±SD*	*Female (%)*	*Total (%)*
Sample size n (%)	92 (76.67%)	28 (23.33%)	120 (100%)	85 (70.83%)	35 (29.16%)	120 (100%)
Age (Mean ±SD)	53.9 ± 12.3	54.4 ± 10.4	57 ± 7.63	43.7 ± 14.1	43.9 ± 13.5	48 ± 9.31
BMI (kg/m^2)	22.66 ± 1.02	25.17 ± 0.11	24.75 ± 0.09	23.84 ± 1.02	24.96 ± 1.02	24.57 ± 1.32
SBP (mm Hg)	128.09 ± 0.18	121.13 ± 0.30	122.27 ± 0.85	112.09 ± 0.18	120.13 ± 0.30	118.27 ± 0.85
DBP (mm Hg)	89.63 ± 0.86	82.34 ± 0.58	84.63 ± 0.18	81.63 ± 0.65	79.34 ± 0.58	80.63 ± 0.75

n: Sample Size

rs121909797) was found to be compatible with the Hardy-Weinberg equilibrium expectation for both cases and controls.

The studied genetic polymorphisms among VDR, CYP24A1, and KL have provided significant results, which correlate the gene frequencies associated with the pathogenesis of CKD. Among the studied gene polymorphisms, VDR rs121909800 polymorphism is found to have a significant association in genotype frequency for alleles GG (60%), GA (28.33%), and AA (11.67%) among the patients, compared to the control subjects which is GG (58.33%), GA (37.50%), and AA (4.17%). It is correlating significantly in patient samples, that the mutant allele AA has a *p*-value of 0.03 (Table 4). It is a statistically significant correlation result among the studied polymorphisms. In addition, the allele carriage frequency analysis reveals G allele carriage has significant results of 95.83 percent with a *p*-value of 0.03. All the observed results of each SNP were also correlated with the predicted Odds Ratio (OR) value (Table 4). In VDR Genotyping for rs121909797 polymorphism, the researchers could not find any statistically significant results in any alleles. Although, the C allele carriage has more than 90 percent compared to other genotypes. In summary, the other variant in CYP24A1 and KL gene shows no significant association with CKD pathogenesis (Tables 5 and 6).

Combined Analysis and Haplotype Distribution

The synergistic effect of VDR, CYP24A1, and KL genotypes was evaluated between the mutant

Table 4: Genotype and allele frequency distribution of VDR gene among CKD patients and control subjects

Genotype & Alleles	*Patients n= 120(%)*	*Control n= 120(%)*	*p –value*	*OR (95% CI)*
		VDR Genotyping for rs121909800 polymorphism		
GG	72 (60.00)	70 (58.33)	0.79	1.07 (0.64 - 1.79)
GA	34 (28.33)	45 (37.50)	0.13	0.65 (0.38 - 1.13)
AA	14 (11.67)	5 (4.17)	0.03*	3.03 (1.05 - 8.72)
		Allele frequency		
Allele G	178 (74.16)	185 (77.08)	0.45	0.85 (0.56 - 1.29)
Allele A	62 (25.84)	55 (22.92)		
		Allele carriage frequency		
Gallele carriage	106 (88.33)	115 (95.83)	0.03*	0.32 (0.11 - 0.94)
Aallele carriage	48 (40.00)	50 (41.67)	0.79	0.93 (0.55 - 1.56)
		VDR Genotyping for rs121909797 polymorphism		
CC	73 (60.83)	76 (63.33)	0.42	0.80 (0.47 - 1.36)
CA	31 (25.83)	32 (26.67)	0.55	1.18 (0.66 - 2.10)
AA	16 (13.34)	12 (10.00)	0.67	1.18 (0.52 - 2.68)
		Allele frequency		
Allele C	177 (73.75)	184 (76.67)	0.45	0.85 (0.56 -1.29)
Allele A	63 (26.25)	56 (23.33)		
		Allele carriage frequency		
Callele carriage	104 (86.67)	108 (90.00)	0.42	0.72 (0.32 - 1.60)
Aallele carriage	47 (39.17)	44 (36.67)	0.68	1.11 (0.66 - 1.87)

Note: *Significant value ($p < 0.05$); OR- Odds Ratio; CI-Confidence Interval

Table 5: Genotype and allele frequency distribution of CYP24A1 gene among CKD patients and control subjects

Genotype & Alleles	*Patients n= 120(%)*	*Control n= 120(%)*	*p –value*	*OR(95% CI)*
		CYP24A1 Genotyping for rs387907322 polymorphism		
CC	82 (68.33)	77 (64.16)	0.78	0.92 (0.52 - 1.62)
C T	30 (25.0)	38 (31.66)	0.25	0.71 (0.40 - 1.26)
T T	8 (6.67)	5 (4.18)	0.39	1.64 (0.52 - 5.17)
		Allele frequency		
Allele C	194 (80.83)	192 (80.00)	0.81	1.05 (0.67 - 1.65)
Allele T	46 (19.67)	48 (20.00)		
		Allele carriage frequency		
C allele carriage	112 95.33)	115 (95.83)	0.39	0.60 (0.19 - 1.91)
T allele carriage	38 (31.67)	43 (35.83)	0.49	0.82 (0.48 - 1.41)
		CYP24A1 Genotyping for rs114368325 polymorphism		
GG	74 (61.67)	81(67.50)	0.34	0.77 (0.45 - 1.31)
GA	34 (28.33)	31(25.83)	0.66	1.13 (0.64 - 2.00)
AA	12 (10.00)	8 (6.67)	0.35	1.55 (0.61 - 3.95)
		Allele frequency		
Allele G	182 (75.83)	193 (80.41)	0.22	0.76 (0.49 - 1.18)
Allele A	58 (21.66)	47 (19.58)		
		Allele carriage frequency		
G allele carriage	108 (90.00)	112 (93.33)	0.35	0.64 (0.25 to 1.63)
A allele carriage	46 (38.33)	39 (32.50)	0.34	1.29 (0.75 - 2.19)

Note: OR- Odds Ratio; CI-Confidence Interval

Table 6:Genotype and allele frequency distribution of KL gene among CKD patients and control subjects

Genotype & Alleles	*Patients n= 120(%)*	*Control n= 120(%)*	*p -value*	*OR(95% CI)*
		KL Genotyping for rs9536314 polymorphism		
T T	78 (65.0)	74 (61.67)	0.59	1.15 (0.68 - 1.95)
TA	31 (25.84)	40 (33.33)	0.20	0.69 (0.39 - 1.21)
AA	11 (9.16)	6 (5.0)	0.21	1.91 (0.68 - 5.36)
		Allele frequency		
Allele T	187 (77.92)	188 (78.33)	0.91	0.97 (0.63 - 1.50)
Allele A	53 (22.08)	52 (21.67)		
		Allele carriage frequency		
T allele carriage	108 (90.00)	114 (95.00)	0.14	0.47 (0.17 - 1.30)
A allele carriage	42 (35.00)	46 (38.33)	0.5922	0.86 (0.51 - 1.46)
		KL Genotyping for rs649964 polymorphism		
T T	67 (55.84)	74 (61.67)	0.3590	0.78 (0.46 - 1.31)
TA	40 (33.33)	38 (31.67)	0.7828	1.07 (0.62 - 1.85)
AA	13 (10.83)	8 (6.66)	0.25	1.70 (0.67 - 4.26)
		Allele frequency		
Allele T	174 (72.50)	186 (77.50)	0.20	0.76 (0.50 - 1.15)
Allele A	66 (27.50)	54 (22.50)		
		Allele carriage frequency		
T allele carriage	107 (89.16)	112 (93.33)	0.25	0.58 (0.23 - 1.47)
A allele carriage	53 (44.17)	46 (38.33)	0.35	1.27 (0.76 - 2.12)

Note: OR- Odds Ratio; CI-Confidence Interval

and heterozygous types compared to the wild type. Patients and control subjects with a combined VDR genotype showed a significant difference in frequency distribution (Table 7). In the CKD patients, thers121909800 polymorphisms showed a higher risk (p = 0.03, OR = 3.037 and 95% CI = 0.05-1.56). The A/C haplotype was found to be more prevalent in patients (7.50%) than in controls (1.66%), and the variation was found to be statistically significant (p = 0.005, OR = 4.50, and 95% confidence interval: CI = 0.95-21.26) (Table 8). The distribution of VDR, CYP24A1 and KL polymorphisms is shown in Table 8.

Table 7: VDR, CYP24A1, and KL gene polymorphisms distribution (dominant and recessive model) in patients and control subjects

SNP	*Model*	*Test*	*Case*	*Control*	*OR*	*95%CI*	*p-Value*
rs121909800	AA+GA VS GG	DOM	48/72	50/70	0.933	0.55 -1.56	0.792
	AA VS GA+GG	REC	14/106	5/115	3.037	1.05 - 8.72	0.038*
rs121909797	AA+CA Vs CC	DOM	47/73	44/76	1.112	0.66 - 1.87	0.689
	AA Vs CA+AA	REC	16/104	12/108	1.384	0.62 - 3.06	0.422
rs387907322	TT+CT Vs CC	DOM	38/82	43/77	0.829	0.48 - 1.41	0.495
	TT Vs CT+CC	REC	8/112	5/115	1.642	0.52- 5.17	0.396
rs114368325	AA+GA Vs GG	DOM	46/74	39/81	1.291	0.75 - 2.19	0.345
	AA VS GA+GG	REC	12/108	8/112	1.555	0.61 - 3.95	0.353
rs9536314	AA+TA Vs TT	DOM	42/78	46/74	0.866	0.51 - 1.46	0.592
	AA Vs TA+TT	REC	11/109	6/114	1.917	0.68 - 5.36	0.214
rs649964	AA+TA Vs TT	DOM	53/67	46/74	1.272	0.76 - 2.12	0.359
	AA Vs TA+TT	REC	13/107	8/112	1.700	0.67 - 4.26	0.257

Table 8: Haplotype distribution of VDR, CYP24A1, KL gene polymorphisms among CKD patients and controls

Haplotype	*Patientsn= 120(%)*	*Controlsn= 120(%)*	*p-Value*	*OR(95% CI)*
		rs121909800 and rs121909797 polymorphisms		
G/A	7 (5.83)	8 (6.66)	0.78	0.86 (0.30 - 2.47)
A/C	9 (7.50)	2 (1.66)	0.05*	4.50 (0.95 - 21.26)
G/C	49 (40.83)	46 (38.33)	0.69	1.11 (0.66 - 1.86)
A/A	4 (3.33)	1 (0.83)	0.20	4.10 (0.45 - 37.26)
		rs387907322 and rs114368325 polymorphisms		
C/A	11 (9.16)	4 (3.33)	0.07	2.92 (0.90 - 9.46)
T/G	5 (4.16)	2 (1.66)	0.26	2.56 (0.48 - 13.48)
C/G	44 (36.66)	47 (39.16)	0.68	0.89 (0.53 - 1.51)
T/A	2 (1.66)	0 (0)	0.29	5.08 (0.24 - 107.03)
		rs9536314 and rs649964 polymorphisms		
T/A	11 (9.16)	4 (3.33)	0.07	2.92 (0.90 - 9.46)
A/T	5 (4.16)	2 (1.66)	0.26	2.56 (0.48 - 13.48
T/T	44 (36.66)	47 (39.16)	0.68	0.89 (0.53 - 1.51)
A/A	2 (1.66)	0 (0)	0.29	5.08 (0.24 - 107.03)

MDR Analysis

The MDR finding analysis was conducted to identify the cases and controls of gene-gene interaction. MDR revealed the results for the two or more possible polymorphisms and their combinations were studied to predict CKD risk. The interaction of the rs121909800, rs649964 polymorphisms has a strong association, and rs387907322, rs9536314 were the best model, with a precision score of 0.77, accuracy of 0.8, and a CV consistency of 10/10 (Fig. 2). The researchers used a threshold value of 1.0 (120/120) to determine the high-risk (dark grey), low-risk (light grey) and blank (empty cell) genotypic combinations in the current data.

Among the studied polymorphisms, rs114368325 was found to be an independent variant, which interacts with the disease association alone and did not reveal any connection with other genes (s 2). The circled figure depicts the association of each gene polymorphism between them. Figure 3 shows different colours such as blue, orange (strong association), and green (midline association). The bold line indicates the stronger interaction, the shorter the line (weak) as connecting qualities. The line's colour shows the kind of interaction. Red indicates a synergistic link (epistasis), yellow shows independence, green is redundancy and blue indicates association (Fig. 3).

Genetic Power Test

The VDR polymorphism genetic power analysis revealed that allele 'A' had 0.05 type 1 error

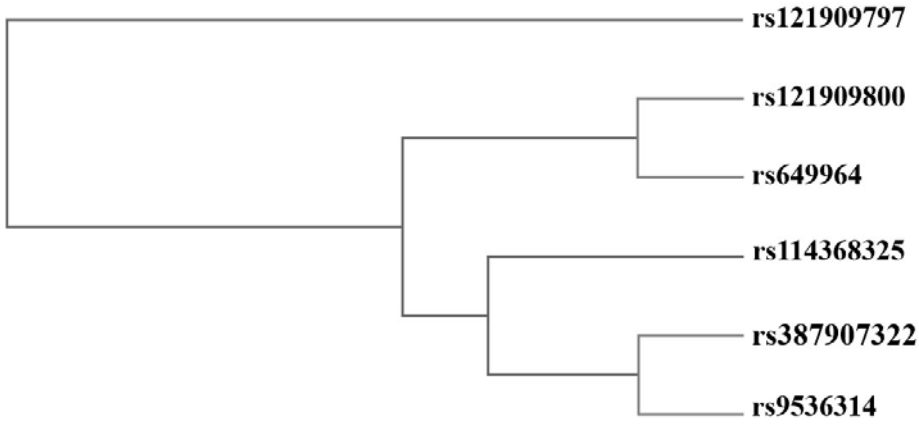

Fig. 2.Graphical model of the MDR analysis of all the six polymorphisms in the view of dendogram

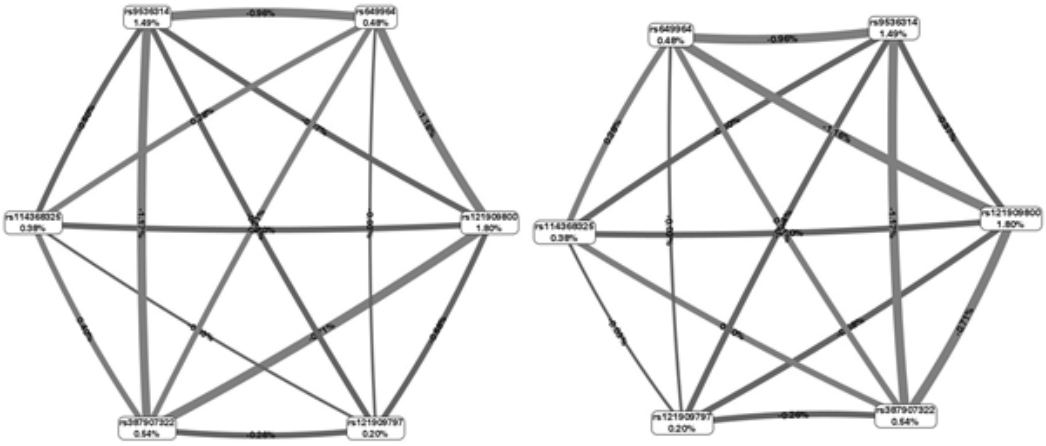

Fig. 3. Circle graph (Left) and Fruchterman-Rheingold graph (Right) retrieved from MDR analysis to correlate the association of studied polymorphisms

between 120 cases and controls. Then it is shown that more than 80 percent of genetic power estimation between 120 cases and 120 controls detected the linkage of CKD and VDR variants in the case studied population.

DISCUSSION

Genetic polymorphisms contribute to the onset of CKD, a disease influenced by several factors. The number of gene polymorphisms associated with CKD differed between ethnic groups. A genetic makeup and selective pressure in these populations may be responsible for these variations. There are a number of serious complications associated with CKD, such as cardiac problems and osteoporosis. The majority of the affected population is older individuals (over 50 years of age). The researchers found that the mean age of male CKD subjects in the study was 53.90 + 12.33 years and the mean age of females was 54.40 + 10.42 years. Among the different gene polymorphisms of VDR, CYP24A1 and KL genes were significantly associated in some studies. The rs121909800, rs121909797, rs387907322, rs114368325, rs9536314 and rs649964 polymorphisms has a

pathogenic role and are involved in vitamin D dysregulation related deficiencies. In the study, the researchers found that VDR rs121909800 polymorphism has a significant association in genotype frequency for alleles GG (60%), GA (28.33%), and AA (11.67%) among the patients compared to the control subjects with GG (58.33), GA (37.50%), and AA (4.17%). As a result, the VDR gene polymorphism rs121909800 is marked as a gene of interest and has a significant association with CKD patients. Haplotype distribution revealed that the rs121909800 polymorphisms are statistically significant ($p = 0.005$, OR = 4.50 and 95% CI = 0.95-21.26). The KL and CYP24A1 haplotype distribution does not show any significance, while the MDR analysis shows rs114368325 was found to have a strong association with rs649964 and rs387907322 polymorphisms. The study contains limitations, including a small sample size and the simple fact that the researchers did not include more patients from different kidney centres to better understand the role of the above-mentioned polymorphism. As part of the study, there are some limitations to heterogeneity in chronic kidney disease. In spite of this, since the samples are derived from homogenous genetic backgrounds, they are unlikely to be influenced by non-measured factors resulting from population stratification. A significant increase in rs121909800 is seen in patients with CKD in the Tamil Nadu population, suggesting a deteriorating effect of genotype on the progression of the disease.

CONCLUSION

The current study discusses the role of genetic variations and their impact on CKD patients. Based on previous studies, this investigation from the computational analysis, the researchers found that the following six SNPs (rs121909800, rs12190979, rs387907322, rs114368325, rs9536314, and rs649964) are showing the promising deleterious effect on CKD patients, and further the researchers correlate the clinical significance association of six different genetic variants of the VDR, CYP24A1, and KL genes. The above said genetic variations were genotyped by ARMS-PCR. These findings are also helpful for the early detection of CKD individuals. The statistical significance was observed for the gene VDR rs121909800 polymorphism. MDR study revealed that rs121909800, rs649964, rs9536314, and rs387907322 have a stronger connection among the six genes investigated. The rs114368325 variation shows statistical association with CKD independently and it could be an important variant in the pathophysiology of CKD but it should be confirmed using a larger number of sample sizes.

RECOMMENDATIONS

The studied polymorphism from the three potential genes associated with CKD pathogenesis concludes that variants have significant changes in the allele frequency among the studied population. Further, for better management of CKD, primary screening of these mutations could be helpful for better and early diagnosis for CKD.

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AUTHOR CONTRIBUTIONS

JMJ analyzed and interpreted the patient data regarding the CKD and was a major contributor in writing the manuscript. KM conceptualized the work design. All authors read and approved the final manuscript.

REFERENCES

Bikle D 2021. Vitamin D: Production, Metabolism and Mechanisms of Action. Endotext. South Dartmouth. United States of America. From <https://www. ncbi.

nlm.nih.gov/books/NBK278935/> (Retrieved on 31 December 2021).
Cañadas-Garre M, Anderson K, Cappa R et al. 2019. Genetic susceptibility to chronic kidney disease–some more pieces for the heritability puzzle. *Frontiers in Genetics*, 10: 453.
Corredor Z, Filho MI, Rodríguez-Ribera L et al. 2020. Genetic variants associated with chronic kidney disease in a Spanish population. *Scientific Reports*, 10(1): 144. https://doi.org/10.1038/s41598-019-56695-2
Donate Correa J, Martín Núñez E, Martínez Sanz R et al. 2016. Influence of Klotho gene polymorphisms on vascular gene expression and its relationship to cardiovascular disease. *Journal of Cellular and Molecular Medicine*, 20(1): 128-133.
Drüeke TB 2021. Hyperparathyroidism in Chronic Kidney Disease. Endotext [Internet]. From <https://www.ncbi.nlm.nih.gov/books/NBK278975/> (Retrieved on 18 October 2021).
Fuhrman BJ, Freedman DM, Bhatti P et al. 2013. Sunlight, polymorphisms of Vitamin D-related genes and risk of breast cancer. *Anticancer Research*, 33(2): 543-551.
Hanson K, Fisher K, Hooper NM 2021. Exploiting the neuroprotective effects of α-klotho to tackle ageing- and neurodegeneration-related cognitive dysfunction. *Neuronal Signaling*, 5(2): NS20200101.
Hill NR, Fatoba ST, Oke JL et al. 2016. Global prevalence of chronic kidney disease-a systematic review and meta-analysis. *PloS One*, 11(7): e0158765.
Hoseinkhani Z, Rastegari-Pouyani M, Tajemiri F et al. 2021. Association of vitamin D receptor polymorphisms (FokI (Rs2228570), ApaI (Rs7975232), BsmI (Rs1544410), and TaqI (Rs731236)) with gastric cancer in a Kurdish population from west of Iran. *Reports of Biochemistry & Molecular Biology*, 9(4): 435.
Hyder R, Sprague SM 2020. Secondary hyperparathyroidism in a patient with CKD. *Clinical Journal of the American Society of Nephrology*, 15(7): 1041.
Imani D, Razi B, Khosrojerdi A et al. 2020. Vitamin D receptor gene polymorphisms and susceptibility to urolithiasis: A meta-regression and meta-analysis. *BMC Nephrology*, 21: 1-18.
Jayaraj JM, Kuriakose BB, Alhazmi AH et al. 2021. Structural and functional insights on vitamin D receptor and CYP24A1 deleterious single nucleotide polymorphisms: A computational and pharmacogenomics perpetual approach. *Cell Biochemistry and Function*, 39(7): 874-885.
Karthikeyan M, Shridevi V, Rose R et al. 2013. Angiotensin gene polymorphisms (T174M and M235T) are significantly associated with the hypertensive patients of Tamil Nadu, South India. *International Journal of Human Genetics*, 13(4): 201-207.
Kongsbak M, Levring TB, Geisler C et al. 2013. The vitamin d receptor and T cell function. *Frontiers in Immunology*, 4: 148.
Laitman Y, Kuchenbaecker KB, Rantala J et al. 2012. The KL-VS sequence variant of Klotho and cancer risk in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Research and Treatment*, 132(3): 1119.
Li S, Zhan J, Wang Y et al. 2019. Association between renal function and bone mineral density in healthy postmenopausal Chinese women. *BMC Endocrine Disorders*, 19(1): 1-7.
Singh KD, Jajodia A, Kaur H 2015. Renin angiotensin system gene polymorphisms in response to antihypertensive drugs and visit-to-visit blood pressure variability in essential hypertensive patients. *Current Pharmacogenomics and Personalized Medicine*, 12(4): 227-235.
Solache-Berrocal G, Rolle-Sóñora V, Martín-Fernández N et al. 2021. CYP24A1 and KL polymorphisms are associated with the extent of vascular calcification but do not improve prediction of cardiovascular events. *Nephrology Dialysis Transplantation*, 36(11): 2076–2083. https://doi.org/10.1093/NDT/GFAA240
Yokoyama S, Oguro R, Yamamoto K et al. 2019. A klotho gene single nucleotide polymorphism is associated with the onset of stroke and plasma klotho concentration. *Aging (Albany NY)*, 11(1): 104. https://doi.org/10.18632/AGING.101728
Zhang L, Yin X, Wang J et al. 2018. Associations between VDR gene polymorphisms and osteoporosis risk and bone mineral density in postmenopausal women: A systematic review and meta-analysis. *Scientific Reports*, 8(1). https://doi.org/10.1038/S41598-017-18670-7

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