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Association of *FokI* polymorphism of vitamin D receptor gene with vitamin D and parathyroid hormone levels in stage IV-V chronic kidney disease patients

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Most of the chronic kidney disease (CKD) stage IV-V patients have deficiency of vitamin D. Active vitamin D binds to vitamin D receptor (VDR) to regulate target gene transcription and mediates diverse biological functions. Various genetic variations such as *FokI* polymorphism, may affect the VDR gene expression and may play an important role in pathogenesis of CKD. In this context, here, we looked into the association of *FokI* polymorphism of VDR gene with serum vitamin D and PTH levels in stage IV-V CKD patients. A total of 150 patients of CKD stage IV-V, aged 25-60 years, and 150 healthy controls (HC), age and sex matched, were enrolled for the study. *FokI* polymorphism was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in the study subjects. The prevalence of different genotypes and allelic frequency distributions were compared in study subjects. Serum PTH and vitamin D were estimated by eCLIA method. No significant differences in genotype and in allelic frequencies between CKD patients and HC were observed. No significant differences in biochemical parameters based on genotypic variations were observed. The results suggest no association of VDR *FokI* polymorphism with CKD. No significant association was noticed between *FokI* variants and PTH or VitD.

Keywords: Calciferol, Intact parathyroid hormone (iPTH), Restriction fragment length polymorphism (RFLP), single nucleotide polymorphisms (SNP)

Chronic kidney disease (CKD) is an important public health problem globally and in India[1,2]. People with CKD often progress to end-stage renal disease (ESRD) requiring renal replacement therapy in the form of dialysis or transplantation[3]. Almost 35% patients of CKD have deficiency in vitamin D. The prevalence of vitamin D deficiency increases with progression of CKD and approaches to 80% in stage IV-V CKD patients[4].

Vitamin D (calciferol) is a pre hormone, obtained through the diet or via skin synthesis. It is subsequently activated through several sequential steps. The 7-dehydrocholestrol in the skin on exposure to sunlight (UV-B rays) is converted to pre vitamin D3 in upper layer of the skin and undergoes non-enzymatic conversion to vitamin D3 (cholecalciferol) in lower layer of the skin and quickly transported to adipose tissue for storage and to liver for hydroxylation to form 25-hydroxyvitamin D3 under the influence of several cytochrome P450 enzyme (CYP2R1, CYP27A1)[5]. Kidney plays a major role in hydroxylation of 25-hydroxyvitamin D3 and plant-based vitamin D2 into active vitamin D3 (1alfa, 25-dihydroxyvitamin D3) under the action of 1-alfa-hydroxylase and cytochrome P450 enzyme (CYP27B1)[6]. In chronic kidney disease, impaired production of 1, 25-dihydroxyvitamin D3 is the principal factor that causes calcium deficiency, secondary hyperparathyroidism, marked by increased intact parathyroid hormone (iPTH) level and further leads to bone disease and other complications[7].

Various single nucleotide polymorphisms (SNP) have been described in the VDR gene, among several allelic variants of the gene encoding VDR, recognized by *ApaI* (allele A/a), *BsmI* (allele B/b), *FokI* (allele F/f) and *TaqI* (allele T/t) restriction endonucleases, some of which may affect the structure or expression of the VDR and thus may play an important role in pathogenesis of CKD[8]. Amongst the various SNPs described, the *FokI* polymorphism (rs2228570, located on chr:12; 47879112 on assembly GRCh38) is a C/T transition polymorphism (ACG to ATG) at the first of two potential translation initiation sites in exon II and is the only exonic SNP described in this gene.

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This 'C' (F allele) to 'T' (f allele) transition results in the generation of a protein shortened by three amino acids, translated from the downstream ATG codon. This transition also results in the loss of the *FokI*-recognition site.

The 'C' allele designated as "F," gives rise to the variant protein, which is more efficient in mediating vitamin D action while 'T' allele designated as "f," gives a truncated protein with three fewer amino acids. This may be one of the potential molecular mechanisms by which *FokI ff* genotype carriers are likely to be associated with different disease condition including increased susceptibility of bone disorders, especially in chronic kidney disease (CKD)[9-11].

Vitamin D plays an important regulatory role on PTH, which is an important mediator of various complications in CKD. This is mediated through the VDR, where the main effect of vitamin D is to decrease PTH gene transcription and inhibit parathyroid cell proliferation, leading to a decrease in serum PTH levels[12]. The relationship between VDR gene polymorphism and the risk of CKD or its complications are not yet well-established. However, variants of the VDR gene have been linked to an increased risk of CKD in some studies[13]. These variations may lead to an altered response to vitamin D, which could affect the functioning of the kidneys. Additionally, VDR polymorphisms could lead to an increase in the renal expression of inflammatory cytokines, which are associated with CKD progression[14,15]. Furthermore, VDR polymorphisms may also lead to increased levels of fibroblast growth factor 23, a hormone that plays a role in CKD[16]. Previously, in a meta-analysis[17], VDR *FokI* gene polymorphism was not found to be associated with CKD susceptibility for overall populations, however, the *FokI f* allele, ff genotype and FF genotype were seen to be associated with CKD in Asians.

One recent study among north Indian population has shown that ff of *FokI* is associated with end stage renal disease (ESRD)[13]. The *FokI* polymorphism may affect the function of VDR resulting in altered efficiency of binding to vitamin D, which in turn may affect the production of downstream molecules[18] and it is postulated that VDR polymorphism may affect PTH levels resulting in dysregulation of calcium and phosphate levels[19,20], but not much literature is available in this regard. Hence, we designed this present study to look into the association of *FokI* polymorphism of VDR gene with CKD and serum vitamin D and PTH levels in stage IV-V CKD patients.

Materials and Methods

Patients were recruited from the Nephrology OPD and laboratory work-up was carried out in the Department of Laboratory Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi. Ethical clearance (Reference No.: IECPG-764/30.01.2020) was obtained from the Institute Ethical Committee and written informed consent was obtained from all recruited patients and controls as per institutional guidelines.

A total of 150 patients diagnosed by nephrologists as chronic kidney disease (CKD)-stage IV-V, defined as kidney damage or GFR less than 60 mL/min/1.73 m2 for more than 3 months, as per National Kidney Foundation (NKF) guideline as part of its Kidney Disease Global Outcome Initiative (KDGOI), aged within 25-60 years, who were not on dialysis, were included in the study. About 150 age and sex matched healthy controls, aged 25-60 years, were also recruited. For staging, eGFR was calculated using four variable Modification of Diet in Renal Disease (MDRD) formula[3].

Sample size was estimated using one study from Lucknow, India[21], where the prevalence of *FokI* (*ff*) polymorphism had been reported to be 8.5% in end stage renal disease patients and 2.6% in controls. Assuming an alpha error of 0.5 and beta error of 0.2 and keeping the power of this study at 0.8 the sample size was calculated using an online tool (www.openepi.com) and was found to be 149 for patients and controls each. Hence in the present study, 150 patients and 150 unrelated, healthy, non-pregnant, age and sex-matched controls were recruited.

Seropositive HIV patients, patients with history of autoimmune diseases on steroids; patients with history of medications which may alter vitamin D-PTH axis like antiepileptics, antineoplastics, antibiotics (like clotrimazole), antihypertensives (Nifedipine, Spironolactone), cyproterone acetate; patients on medication such as vitamin D, calcium based phosphate binders; patients with pregnancy, malignancy, tuberculosis, severe sepsis and shock were excluded from the study.

Blood collected in serum separator evacuated blood collection tubes, were kept for 20-30 min at room

temperature (18-24°C) to allow them to clot. These were further centrifuged at 3000 rpm for 10 min for serum separation and were analyzed on automated clinical chemistry and immunoassay analyzer (Cobas c702 and e801, Roche Diagnostics, Germany) for biochemistry parameters i.e., Serum iPTH, serum total vitamin-D, serum calcium, serum phosphorus, serum ALP, serum urea and serum creatinine. DNA was extracted from 200 µL fresh blood collected in EDTA tubes using spin column based commercially available kits (Qiagen, USA)

FokI polymorphism of *VDR* gene

A polymerase chain reaction (PCR) was carried out to identify the genotypes of *FokI* SNP using the following primer pairs; (Sense): 5'-AGCTGGCCCTGG CACTGACTCTGCTCT-3' and (Anti-Sense): 5'-ATGG AAACACCTTGCTTCTTCTCCCTC-3'. Amplification consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension for 72°C for 1 min and final extension at 72°C for 5 min. Amplified products (265 bp) were run on 1.5% agarose check gel. The PCR product was digested with FokI restriction enzyme generating two fragments of 196 and 69 bp each after digestion in the presence of FokI enzyme. The products were then run on a 1.5% agarose gel.

Statistical analysis

Statistical analysis was done with Stata/SE 14.0 (College station Texas, USA). Data with a normal distribution were expressed as mean ± standard deviation (SD). Data with a non-normal distribution were presented as the median and inter-quartile range. The t-test and χ^2 (Chi-squared) test were used to determine differences of quantitative parameters. Independent t-test/Mann-Whitney (Wilcoxon rank sum) test for non-parametric data with two groups and Kruskal-wallis (one way ANOVA) test for nonparametric data with more than two groups were used to compare the available data. Genotype frequencies and Allelic frequency were compared between the groups using the χ^2 test (test for randomness with categorical outcome). (Chi-squared) χ^2 test was also used to determine Hardy–Weinberg equilibrium (HWE). Spearman rank correlation analysis was used to look for any correlation between parameters in CKD Patients and in healthy control (HC) groups. Statistical significance was defined at a p-value less than 0.05 (P <0.05).

Results

This case-control study had 150 CKD patients along with 150 controls. General and baseline clinical characteristics of participants of the study are summarised in Table 1. Table 2 depicts the results of correlation analyses of different biochemical parameters among CKD patients. In CKD patients, significant correlation was observed between iPTH, and calcium, phosphorus and ALP. Other parameters did not reveal significant correlations between different combinations.

FokI polymorphism of *VDR* gene

FokI polymorphism was studied in all the 150 CKD patients and 150 healthy controls (HC). Following DNA isolation and PCR amplification, restriction digestion was done using *FokI* restriction endonuclease on the amplicons and visualised on 1.5% agarose gel electrophoresis. The Restriction Fragment Length Polymorphism (RFLP) was identified with ethidium bromide staining. Homozygous wild-type individuals (FF variants) showed 265 bp fragment, heterozygous mutant

Table 1 — Demographic and baseline clinical characteristics of chronic kidney disease (CKD) patients and Healthy control (HC)

Characteristics	CKD (n=150) (Mean ± SD)	HC (n=150) (Mean ± SD)
Age (yr)	41.0±11.5	35.5±8.2
Gender (M/F)	87/63	107/43
Age interval (25-40) yr	78(52%)	(107)71%
Age interval (41-60) yr	72(48%)	43(29%)
Serum urea (mg/dL)	119.6±53.4	19.6±5
Serum creatinine (mg/dL)	6.5±3.3	0.6±0.1
Serum calcium (mg/dL)	9.3±0.9	8.3±0.73
Serum phosphorus (mg/dL)	5.2±1.7	3.6±0.6
	Median (IQR)	Median (IQR)
Serum ALP (IU/L)	110 (83-154)	85 (73-96)
Serum iPTH (pg/dL)	252 (123-443)	39.8 (25.9-54.1)
Serum total vitamin-D (ng/mL)	16.9 (11.1-25.1)	13.6 (8.3-20.1)

[IQR, Inter quartile range; HC]

Table 2 — Spearman rank correlation analysis between different combinations of biochemical parameters in CKD patients

CKD patients	Correlation co-efficient (r)	Level of significance (p value)
Total vitamin-D and iPTH	–0.1419	0.0831
Total vitamin-D and calcium	0.0894	0.2767
Total vitamin-D and phosphorus	–0.0215	0.7943
Total vitamin-D and ALP	–0.1426	0.0818
iPTH and calcium	–0.3253	0.0001
iPTH and phosphorus	0.2798	0.0005
iPTH and ALP	0.3793	0.0001
Calcium and phosphorus	–0.1192	0.1463
Calcium and ALP	–0.1516	0.0641
ALP and phosphorus	0.1118	0.1732

individuals (Ff variants) showed three bands of 265, 196 and 69 bp and homozygous mutant individuals ("ff" variants) showed bands of 196 and 69 bp as shown in Fig. 1. The distribution of *FokI* polymorphisms in CKD patients and HC are summarized in Table 3.

Genotype frequencies were compared between CKD and HC using chi squire (χ^2) test for randomness of data with categorical outcome. Allele frequencies were calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. Genotype frequencies of both CKD patients and HC were tested to determine whether observed genotype frequencies are consistent with Hardy–Weinberg equilibrium and were found to be up to the required standard. No significant differences in genotype and in allelic frequencies between CKD patients and HC were observed.

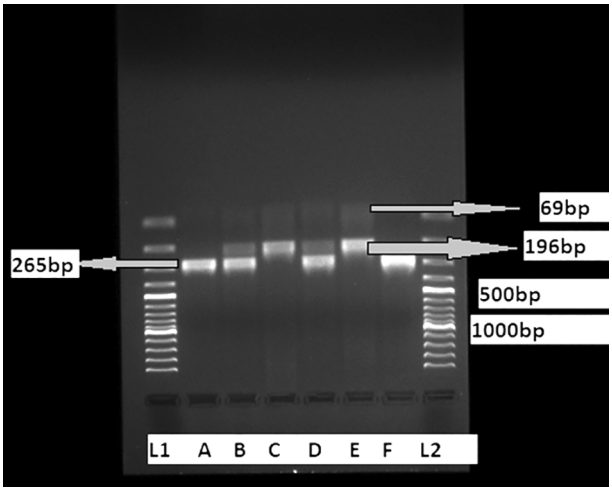

Fig. 1 — RFLP-gel image of Fok1 variant of VDR gene polymorphism in study population. [L1 and L2: Ladder. In Lanes: A and F, Undigested PCR product (band of 265 bp) with Fok1 restriction endonuclease enzyme; represents FF genotype. Ff variants are observed in lanes B and D; displays three bands of 265, 196 and 69 bp. "ff" variants are observed in lanes C and E display bands of 196 and 69 bp]

Table 3 — Distribution of *FokI* polymorphism in CKD patients and HC

FokI polymorphism	CKD (n=150)	HC (n=150)
FF	80 (53.33 %)	86 (57.33%)
Ff	62 (41.33 %)	56 (37.33%)
ff	8 (5.33 %)	8 (5.33%)

Table 4 — Evaluation of Biochemical parameters based on genotypic variation

CKD (n=150)	FF (80)	Ff (62)	ff (8)	*p* value
iPTH	273.5 (119-483)	238 (141-343)	378.5 (219-474)	0.429
Vitamin D	14.9 (10.8-24.8)	18.75 (11.3-24.8)	17.45 (10-30)	0.652
Calcium	9.3±0.97	9.34±0.84	9.5±0.9	0.49
ALP	110 (83-149)	114 (85-161)	101 (81-153)	0.932
Phosphorous	5.23±0.1.86	5.17 ±1.59	4.76 ±1.38	0.334
HC (n=150)	FF (86)	Ff (56)	ff (8)	*p* value
iPTH	38.4 (25.9-53.5)	38.85 (25.9-56)	42.25 (28-46)	0.245
Vitamin D	13(7.8-20)	14.1(8.5-20)	14.3(12-15.8)	0.977
Calcium	8.18±0.81	8.34±0.6	8.16±0.7	0.600
ALP	83(71-94)	85.5(75-96)	87(80-95)	0.329
Phosphorous	3.69±0.64	3.59±0.63	3.72±0.75	0.821

Evaluation of biochemical parameters based on genotypic variation

No significant differences in biochemical parameters based on genotypic variations in CKD patients and in HC were observed as shown in Table 4.

On further subgroup analysis, we observed a remarkable finding in ff genotype group in CKD patients. Median value of iPTH was found to be markedly increased in ff genotype [378.5 (219-474)] than that of in FF genotype [273.5 (119-483)] in CKD patients. But this does not translate into statistical significance probably due to wide variation of iPTH levels observed in FF subgroups. In few CKD patients with FF genotype, very high iPTH level (Approx., 2000 pg/mL) was observed. However, most of the patients in this group had an iPTH level less than 1000 pg/mL.

Discussion

Chronic kidney disease (CKD) demands attention as it often progresses to end-stage renal disease (ESRD) requiring renal replacement therapy in the form of dialysis or transplantation. Its progress is linked to vitamin D deficiency, a prehormone, which is synthesised in the skin and subsequently activated by several sequential steps in the liver and the kidney to produce 1, 25-dihydroxyvitamin D3. Insufficient activation results in calcium deficiency, leading to compensatory increase in PTH level causing secondary hyperparathyroidism[5]. Furthermore, vitamin D mediates its action in the body through the VDR. Active vitamin D3 binds to VDR to regulate target gene transcription helping in calcium uptake or bone formation and other diverse biological functions such as regulation of calcium-phosphorus metabolism, immunity and inflammatory state[6,7].

Several allelic variants of the gene encoding VDR, have been recognized by *ApaI* (allele A/a), *BsmI* (allele B/b), *FokI* (allele F/f) and *TaqI* (allele T/t) restriction endonucleases[8]. *FokI* restriction site is located in the initiation codon region of VDR, which is the only functional locus known to affect VDR protein peptide chain structure. Though *FokI*

polymorphism is associated with different disease conditions including CKD[9-11], ethnic differences in VDR *FokI* polymorphism prevalence have also been reported in the literature.

Here, we studied the association of *FokI* polymorphism of the VDR gene in stage IV-V CKD patients. Furthermore, total vitamin D and parathyroid hormone (iPTH) levels were measured in these patients and their association with *FokI* VDR polymorphism was studied. We compared the genotype frequencies and allelic frequency between CKD and HC. Genotype frequencies of both CKD patients and HC were tested to determine whether observed genotype frequencies are consistent with Hardy–Weinberg equilibrium. Significant difference in genotypic frequency was not observed between expected and observed allele frequencies [observed: FF (80), Ff (62), ff (8) *vs*. expected: FF (82), Ff (58), ff (10)]. In present study, genotype frequencies in CKD patients were observed as: FF-53% (n=80), Ff-42% (n=62), ff-5% (n=8) and in HC as: FF-57% (n=86), Ff- 38% (n=56), ff-5% (n=8) and allelic frequencies in CKD patients were observed as: F-74% and f-26% and in HC as F-76% and f-24%. No significant differences in genotype and in allelic frequencies between CKD patients and HC were observed. Hence, no association was observed for *FokI* variants with CKD patients as compared to controls. Serum calcium and phosphorus levels were observed to be higher in CKD patients than the controls. Significantly increased levels of iPTH were seen in CKD group as compared to HC. These results are consistent with previously reported studies. Tripathi *et al*.[13] in their study in North Indian population observed that the ff genotype of *FokI* was present in 8.5% of the ESRD patients, while in controls it was found in 2.6% of the individuals and both the groups differed significantly. The carriers of ff genotypes were reported to be at a higher risk of developing ESRD; this may be due to truncated protein levels, and hence a less efficient vitamin D receptor. The carrier of ff genotypes of *FokI* had a lower iPTH level than those with FF genotypes in the limited number of studied patients. However, Zhou *et al*.[17] in a meta-analysis, found that VDR *FokI* f allele, ff genotype and FF genotype were not associated with CKD risk in overall populations (f allele: OR = 1.08, 95% CI: 0.67– 1.76, ff genotype: OR = 1.15, 95% CI: 0.42–3.14, FF genotype: OR = 1.02, 95% CI: 0.76–1.39) VDR *FokI* f allele, ff genotype and FF genotype were also not associated with CKD risk in Caucasian population.VDR *FokI* f allele, ff genotype and FF genotype were associated with CKD risk in Asian population. Our results show that there is no association of *FokI* variants with CKD in the north-Indian population.

Earlier, Bid *et al*.[22] tried to determine the distribution of VDR gene Fok-I polymorphisms in unrelated normal healthy individuals from a north Indian population. They obtained allelic frequencies of (68.5% *vs*. 31.5%) for (F *vs*. f) alleles, with 44, 49 and 7%, respectively, for genotypes FF, Ff and ff. These results suggest that the frequency and distribution of the polymorphisms in India are substantially different from other populations and ethnic groups. The results of present study are consistent with the meta-analysis published by Zhou *et al*.[17]. The present study's results show no significant association of *FokI* polymorphism with stage IV-V CKD.

Results of correlation analysis, performed between different combinations of biochemical parameters in CKD patients and HC revealed significant negative correlation iPTH and calcium. Significant positive correlation was observed between iPTH and phosphorus and iPTH and ALP. Other parameters did not reveal significant correlations between the different combinations. These observations are consistent with previously published literature.

Biochemical parameters based on genotypic variation in CKD patients and in HC have been studied. However, no significant differences in biochemical parameters were observed in FF, Ff and ff genotypic subgroups in CKD or HC groups. Tripathi *et al*.[13] also reports no significant difference in PTH, calcium and phosphorus levels amongst *FokI* genotypic variants. However, they have not reported Vit D levels in their study.

Overall, the present study findings were consistent with previous studies. However, a remarkable finding in ff genotype group in CKD patients was observed. Median value of iPTH was found to be markedly increased in ff genotype [378.5 (219-474)] than that of in FF genotype [273.5 (119-483)] in CKD patients. But this does not translate into statistical significance probably due to wide variation of iPTH levels observed in FF subgroups. In few CKD patients with FF genotype, very high iPTH level (Approx., 2000 pg/mL) was observed. However, most of the patients in this group had an iPTH level less than 1000 pg/mL.

Since, present study had only 8 patients with ff genotype amongst CKD patients, more studies are required to confirm the association of high iPTH in ff genotypes in CKD. Limitations of the present study are small sample size, possibility of selection bias and confounding factors, single centric and cross sectional study. Only *FokI* gene polymorphism studied in the present study, it can be done in other variant such as *TaqI*, *ApaI*, *BsmI* and Cdx2.

Conclusion

The present study reveals no association of VDR *FokI* polymorphism with chronic kidney disease (CKD). Furthermore, no significant differences in biochemical parameters were observed in FF, Ff and ff genotypic subgroups in CKD or Healthy Control Groups. Further subgroup analysis, based on age group (25-40) year and (41-60) year also revealed no significant differences in biochemical parameters based on genotypic variations in CKD patients and in HC.

Conflict of interest

Authors declare no competing interests.

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