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Genetic Polymorphism in the Vitamin D Receptor Gene and 25-Hydroxyvitamin D Serum Levels in East Indian Women with Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovary syndrome (PCOS) is the most common metabolic abnormality such as changes in lipid profile, diabetes, hypertension and metabolic syndrome occurring in young women of reproductive age. Low vitamin D levels were found to be associated with the development of obesity and insulin resistance in women with PCOS. Variants on vitamin D receptor (VDR) gene have also been related to metabolic comorbidities in general population.

Aim: The aim of this case-control study was to investigate whether the VDR gene polymorphisms are associated with susceptibility to PCOS.

Methods: Women with PCOS and a control group, all aged 16-40 years, were enrolled. Genotyping of VDR Fok-I (rs2228570), VDR Apa-I (rs7975232) as well as GC (rs2282679), DHCR7 (rs12785878) SNPs between groups were determined by using direct sequencing. Serum 25-hydroxyvitamin D [25(OH)] levels were measured by ELISA.

Results: Mean serum 25(OH)D in the PCOS and control samples were 19.08 ± 7 and 23.27 ± 6.03 (p=0.048) which were significantly lower in PCOS patients compared with controls. CC genotype of the VDR Apa-I SNP was same frequent in PCOS (25.6%) and controls (25.6%) (OR: 0.9995; 95%CI: 0.528 to 1.8921; p=0.9987). The CC genotype was also significantly associated with lower E2 (p=0.031) and androstenedione levels (p=0.026). We observed a significant association of GC polymorphism with 25(OH)D levels. PCOS women carrying the GG genotype (in GC genes) had significantly higher risk for vitamin D deficiency than women carrying the TT genotype.

Conclusions: In conclusion, data from this study indicate that vitamin D levels are lower, and vitamin D deficiency more frequent, in PCOS than in controls. The present findings suggest that the Apa-I, Fok-I polymorphism of the VDR gene is associated with PCOS and seems to modulate ovarian steroid secretion. Further studies are needed to better clarify the biological mechanisms by which the polymorphism influences PCOS risk.

Keywords: Vitamin D receptor; Polymorphism; Vitamin D; Polycystic ovary syndrome

Background

It has been speculated that the majority of individuals in the India are deficient in Vitamin D and that Vitamin D deficiency has become an epidemic in our country. There is widespread prevalence of varying degrees (50-90%) of Vitamin D deficiency with low dietary calcium intake in Indian population according to various studies published earlier [1]. A deficiency of Vitamin D not only causes poor bone mineralization but also has been implicated in numerous us chronic diseases. Vitamin D deficiency is common in women with polycystic ovary syndrome (PCOS), with the 67-85% of women with PCOS having serum concentrations of 25-hydroxy vitamin D [25(OH)D] <20 ng/ml. Vitamin D deficiency may intensify symptoms of PCOS, with observational studies showing lower 25(OH)D levels were associated with insulin resistance, ovulatory and menstrual irregularities, lower pregnancy success rate, hirsutism, hyper-androgenism, obesity and elevated cardiovascular disease risk factors [2]. PCOS is among the most common disorders in women of reproductive age and has a strong genetic component [3]. Data on the role of gene variants involved in vitamin D metabolism in PCOS are thin but suggest an association of VDR (Vitamin D Receptor) and vitamin D level related variants with metabolic and endocrine parameters in women with PCOS [4]. Several studies although limited by modest sample sizes have suggested associations between VDR polymorphisms and the development of PCOS as well as insulin resistance [5-8]. Different distributions e.g. of VDR Apa-I and Fok-I gene polymorphisms were found in a cohort of 162 women with PCOS and their controls [6]. Further genes involved in vitamin D synthesis, hydroxylation and transport and their role in PCOS are currently under investigation [4]. It has been reported that vitamin D deficiency reduces mating success and fertility in female rats. Female rats fed a vitamin D deficient diet are capable of reproduction, but overall fertility is decreased by 75%, and litter size is reduced by 10% [9]. Both VDR and 1α-hydroxylase knockout female mice are infertile and present with uterine hypoplasia, impaired folliculogenesis, and anovulation [10-12]. Vitamin D is the key regulating hormone in calcium homeostasis. It has been shown that calcium plays a role in oocyte activation and maturation resulting in the progression of follicular development [13]. In this context, vitamin D and calcium repletion might lead to normalization of menstrual cycles and restoration of ovulation in PCOS women [14]. Several polymorphisms

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(such as Apa-I, Fok-I,) have been described for the vitamin D receptor (VDR) gene, located on chromosome 12 [12q13.11].

VDR mediates most effects of vitamin D on gene expression via formation of a heterodimer with the retinoid× receptor that binds to promoter regions of many target genes [15]. VDR gene polymorphisms have also been associated with vitamin D levels, glucose metabolism, insulin secretion, and peripheral action in different populations [16-18].

GC [Group-Specific Component (Vitamin D Binding Protein)], is the protein encoded by this GC gene (4q13.3) belongs to the albumin gene family. It is a multifunctional protein found in plasma and on the surface of many cell types. It binds to vitamin D and its plasma metabolites and transports them to target tissues. Variations of this gene have linked to defects in Vitamin D bindings and transport them into target tissue.

DHCR7 [7-Dehydrocholesterol Reductase; Protein Coding] is a membrane-bound enzyme that catalyzes the final step of cholesterol biosynthesis encoded by DHCR7 gene (11q13.4). Mutations in the DHCR7 gene have been linked defects in cholesterol biosynthesis.

In this study, we hypothesized that functional SNPs may impact VDR expression and might then be associated with susceptibility to the phenotypes of PCOS. We investigate the association of VDR variants including Apa-I, Fok-I as well as GC and DHCR7 variants with metabolic and endocrine parameters including 25(OH)D levels in PCOS women in an East Indian population. This study may provide more detailed genetic information for PCOS, and to understand the functional consequences of these variations.

Materials and Methods

Subjects, 125 women with PCOS were enrolled, along with 82 women without any evidence of PCOS as a control group; the women of both groups were aged 16-40 years. The subjects were observed for routine gynecological examination at OPD of ILS Hospital, Kolkata, India. This study was reviewed and approved by institutional ethics committee (ECR/130/Inst/WB/2013). Written informed consent was obtained from all participating subjects according to ethical guidelines. Based on the accessible information on the prevalence rate of PCOS and with 95% confidence and 20% allowable error, the minimum sample size was estimated at 120. Inclusion Criteria: 1) Diagnosis of PCOS based on revised Rotterdam criteria 2) Age from 16 to 40 years. Exclusion Criteria: 1) Current pregnancy or nursing 2) Conditions known to affect vitamin D deficiency like renal disease, liver disease, gastro intestinal problems, malnutrition 3) Patients on calcium and vitamin D supplementation.

Biochemical and anthropometric analysis

On the first OPD visit, a detailed history was taken including the age, presenting complaints with duration, detailed menstrual history, past medical history and family history. Anthropometric measurements like weight, height, BMI (body mass index), waist to hip ratio (WHR) were measured. Body Mass Index (BMI) is defined as the weight in kilograms divided by the square of the height in meters (kg/m²). Obesity was defined as a BMI ≥25 kg/m² and non-obese as BMI <25 kg/m². This was based on the consensus statement that the cut-offs for overweight and obesity need to be revised for Asian Indians [19]. For assessing Vitamin D levels, 5 ml of venous blood was collected and centrifuged and serum was stored at -20 degrees in our research lab freezer until analysis was performed. Controls were selected from age matched female doctors, nursing staff, para-medical staff who had regular menstrual periods,

who were non pregnant and not lactating. Those who had chronic illness and who were taking vitamin D preparations were excluded. Vitamin D levels were compared in the PCOS groups and also with the controls. 25OHD was measured using a commercially available ELISA (IDS, Boldon, UK). Chemiluminescent immunoassays (Siemens DPC Buhlmann, Salzburg, Austria) were used to measure serum E2, DHEAS (dehydroepiandrosterone sulphate), androstenedione and total testosterone, with sensitivity (S) of <7.0 pg/ml, 3.0 µg/dl, <0.03 ng/ml, and 0.1 ng/ml, respectively. Insulin (S=0.2 µUI/ml), and 17OHP (S=0.10 ng/ml) were measured with radioimmunoassays (Dia-Sorin, Stillwater, USA; LINCO Research, St. Charles, USA and ICN Biomedicals Inc., Costa Mesa, USA, respectively).

Genotyping

Approximately 10 ml peripheral blood samples were collected from the subjects with the help of the collaborating doctors in an EDTA tube which was used as an anticoagulant. Genomic DNA extracted from fresh whole blood by QIA amp Blood Kit (Qiagen, Hilden, Germany). Genomic DNA used as a template in the polymerase chain reaction (PCR). Molecular genotyping for the VDR Fok-I (rs2228570), Apa-I (rs7975232) as well as GC (rs2282679), DHCR7 (rs12785878) SNPs were performed by PCR. The PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized under UV light. Only those PCR products that had a single amplification product with no evidence of nonspecific amplification were used for DNA sequencing. The PCR products free of contaminating bands due to nonspecific amplification were column purified using a Qiagen PCR-purification kit (Qiagen, Hilden, Germany), and bidirectional sequencing was performed in an ABI Prism 3130 DNA sequencer (Applied Biosystems, Foster City, CA) using dye-termination chemistry. The sequences were analyzed using pairwise BLAST to examine if there were any changes from the normal sequence available in the database.

Statistical analysis

Continuous results are expressed as mean ± SD or median and interquartile range. Comparisons between means were analyzed by the unpaired two-tailed Student's t-test. Categorical variables and the genotypic frequencies from Hardy-Weinberg equilibrium for each SNP were compared using the Pearson chi-square ($x2$) test. Odds ratios (ORs) and 95% confidence interval (95% CI) were obtained using $x2$ unbiased risk estimation. Statistical analyses were performed using the SPSS program (Version 20.0, SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results and Discussion

The mean age of subjects in the study group was 25.78 years in the PCOS group and 26.86 years in the control group. Table 1 shows anthropometric, clinical, and hormonal features with PCOS and controls women. The groups were similar in terms of androstenedione level (p=0.502). Frequency of overweight/obesity was higher in the PCOS group than in controls (p=0.001). Insulin, DHEAS, and 17OHP were significantly higher in PCOS than in controls (p<0.05). Mean serum 25(OH)D levels were significantly lower in PCOS patients compared to controls, 19.08 ± 7 and 23.27 ± 6.03 (p=0.048) respectively.

All four SNP (VDR Apa-I, VDR Fok-I, GC, and DHCR7) were in Hardy-Weinberg equilibrium in both cases and controls. Table 2 shows that genotype distribution of VDR, GC and DHCR7 genes polymorphisms in cases and controls. In table it is described that, the CC genotype of the VDR Apa-I SNP was same frequent in PCOS (25.6%) and controls (25.6%) (OR: 0.9995; 95%CI: 0.528 to 1.8921;

Variable	PCOS women (n=125)	Control Women (n=82)	p value
Age (yrs)	25.78	26.86	0.012
BMI (kg/m²) 0.0001	27.3± 7.5	23.7 ± 4.7	0.001
Estradiol (pg/ml)	16.65 (11.74-39.83)	36.05 (14.75-56.73)	0.37
DHEAS (µg/dl)a	137.89 (79.78-199.90)	86.53 (56.30-117.70)	<0.001
17OHP (ng/ml)a	0.73 (0.56-1.76)	0.59 (0.39-1.92)	0.011
25(OH)D (ng/ml) 0.0001	19.08 ± 7.32	23.27 ± 6.03	0.048
Insulin (µUI/ml)a	17.47 (10.76-21.20)	13.87 (10.68-20.02)	0.509
Androstenedione (ng/ml)	1.65 (0.98-2.38)	1.80 (1.58-2.69)	0.502

Values are expressed as mean ± SD or a median and interquartile range (25-75%). BMI: Body Mass Index; 17OHP: 17hydroxyprogesterone; 25(OH)D: 25-Hydroxyvitamin D.

Table 1: Anthropometric, clinical, and hormonal features of women with PCOS and controls.

Genotype/ Allele	PCOS women	Control Women	p value
VDR Fok-I	(n=125)	(n=82)	
AA	21	28	
AG	67	35	0.016176
GG	37	19	
VDR Apa-I	(n=125)	(n=82)	
AA	23	32	
AC	70	29	0.002096
CC	32	21	
GC	(n=125)	(n=82)	
TT	33	29	
TG	73	43	0.374919
GG	19	10	
DHCR7	(n=125)	(n=82)	
TT	40	29	
TG	65	49	0.049984
GG	20	4	

Table 2: Allelic frequencies of VDR and vitamin D level associated variants.

p=0.9987). The CC genotype was also significantly associated with lower E2 (p=0.031) and androstenedione levels (p=0.026) (Figure 1). In the GC SNP's the GG genotype was slightly higher frequent in PCOS (15.2%) than in controls (12.19%) (OR: 1.2906; 95%CI: 0.5672 to 2.9366; p=0.543004).We observed a significant association of GC SNP polymorphism with 25(OH)D levels in PCOS women (Figure 2). PCOS women carrying the GG genotype (in GC genes) had significantly higher risk for vitamin D deficiency than women carrying the TT genotype. We found the allelic frequencies of different SNPs in PCOS and control women. VDR and DHCR7 genotype frequency distributions deviated significantly from the Hardy-Weinberg equilibrium (p<0.05). There was no difference in genotype frequencies in GC SNP between PCOS and control women (Table 2). In the VDR Apa-I the nucleotide A change to C (AA wild type and CC variant type) and VDR Fok-I the nucleotide A converted to G (AA wild type and GG variant type as shown in Figure 3A and 3B. We also observed a significant association of DHCR7 SNP polymorphism with 25(OH)D levels in PCOS women (Figure 4). Polymorphisms of the VDR gene might be associated with PCOS and biochemical markers related to PCOS. These studies involved the analyses of variants that are located at the 3'end of the VDR gene such as the VDR Fok-I, Apa-I. However, those variants are not likely to influence the function of the VDR itself

because they are in an intron or do not change amino acid sequence. In summary, variants in the GC and DHCR7 gene are associated with serum 25(OH)D levels and VDR Apa-I variants are associated with testosterone levels in PCOS women. Moreover, we confirmed results from previous genome-wide association studies showing an association of GC and DHCR7 polymorphisms with an increased risk for vitamin D deficiency. Nevertheless, those polymorphisms might be important in risk prediction or risk calculation in PCOS women (for GC genotype TT reference, OR=2.46; p=0.008 and for DHCR7 SNP TT reference, OR=2.49; p=0.029 respectively.

Conclusions

In conclusion, to the best of our knowledge, this is the first study to report the VDR as well as GC and DHCR7 gene polymorphism and response on serum 25(OH)D levels in East Indian PCOS women. This study found significantly low serum 25(OH)D levels in homozygous
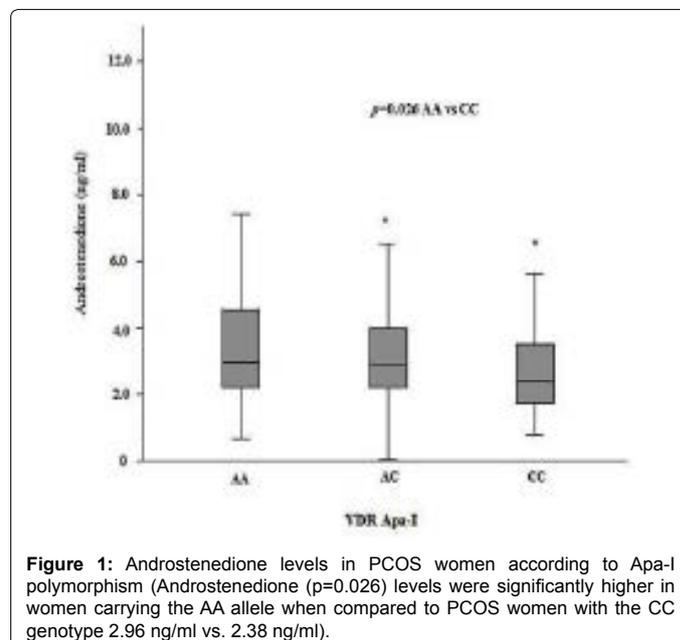

Figure 1: Androstenedione levels in PCOS women according to Apa-I polymorphism (Androstenedione (p=0.026) levels were significantly higher in women carrying the AA allele when compared to PCOS women with the CC genotype 2.96 ng/ml vs. 2.38 ng/ml).
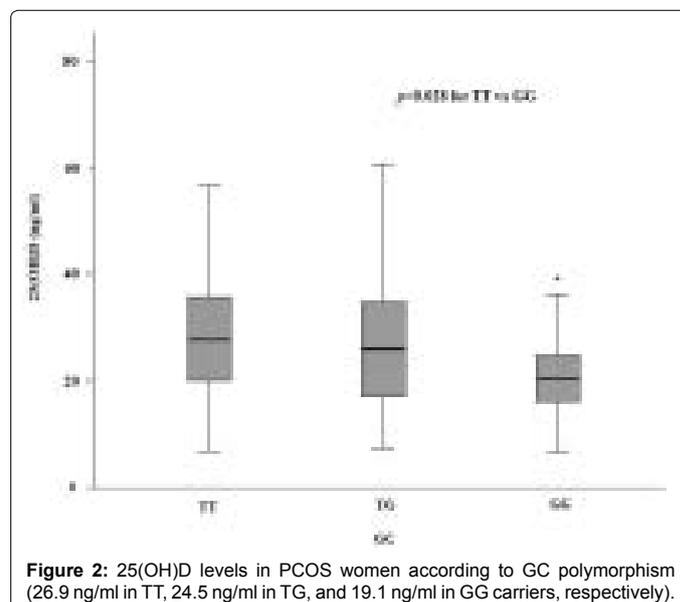

Figure 2: 25(OH)D levels in PCOS women according to GC polymorphism (26.9 ng/ml in TT, 24.5 ng/ml in TG, and 19.1 ng/ml in GG carriers, respectively).
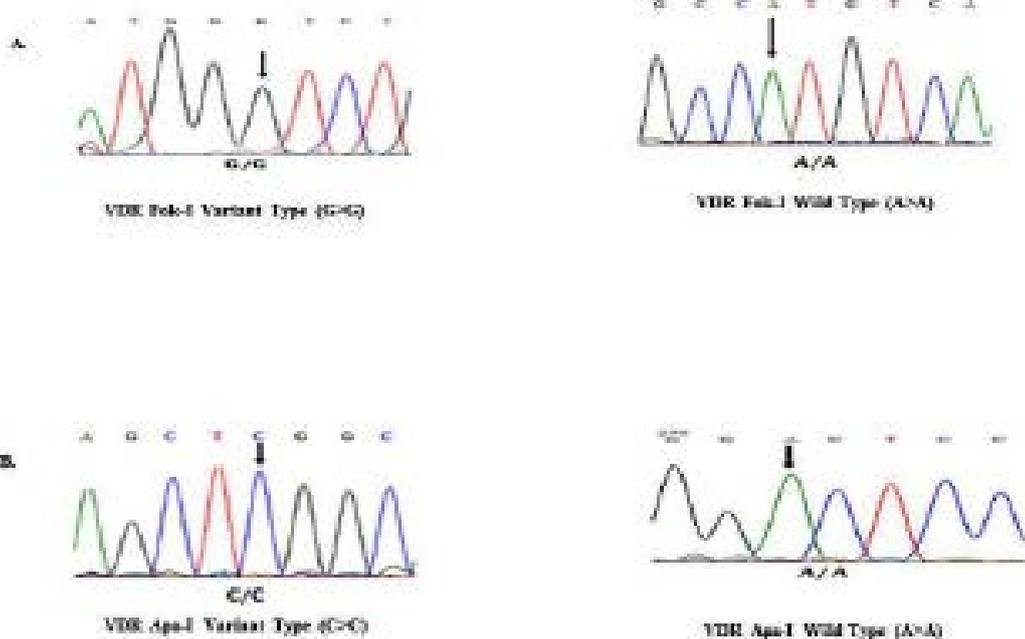
Figure 3: Electropherograms of VDR gene. Arrow indicates the position of nucleotide change (DNA sequencing shows in Fok-I SNP's the wild type allele (AA) converted to variant one (GG) and in Apa-I SNP's the wild type allele (AA) converted to variant one (CC).
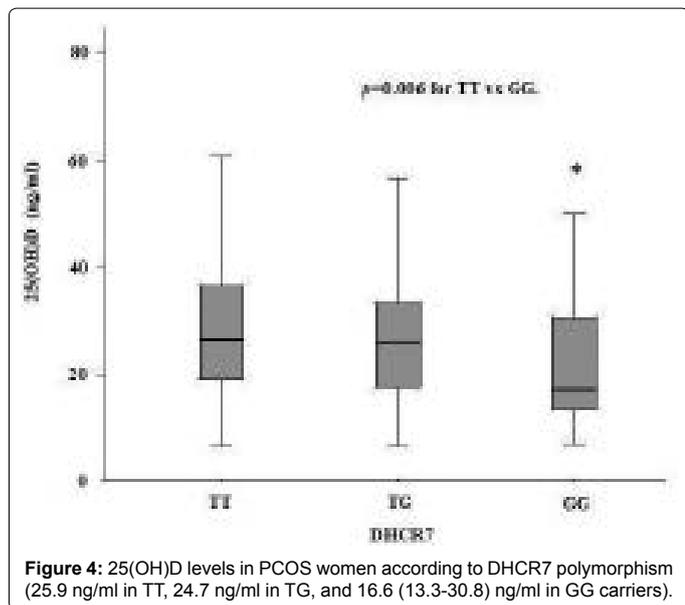

Figure 4: 25(OH)D levels in PCOS women according to DHCR7 polymorphism (25.9 ng/ml in TT, 24.7 ng/ml in TG, and 16.6 (13.3-30.8) ng/ml in GG carriers).

variant alleles of VDR gene polymorphisms in both PCOS groups. A limitation of the study is the small sample size; therefore, we have to be careful in making this conclusion. Further studies using larger sample sizes are necessary to confirm the current findings.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

DS carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. RC helps in the diagnosis of study groups and preparing the manuscript.

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