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RESEARCH ARTICLE

VITAMIN D RECEPTOR GENE POLYMORPHISM IN OBESE AND NON OBESE INDIAN WOMEN WITH POLYCYSTIC OVARY SYNDROME

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ABSTRACT

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of the women in reproductive age group. Accumulating evidences from the recent studies indicate that vitamin D receptor (VDR) genetic variants may influence the development of insulin resistance and so related to the pathogenesis of polycystic ovary syndrome. The aim of the present study was to determine the VDR BsmI gene variant in intron 8 (A/G) (rs1544410) in normal controls, obese PCOS women and non obese PCOS women in India. A total of 225 women aged between 19-36 years participated in the study. The subjects were divided into three groups as obese PCOS women, non obese PCOS women and healthy controls and each group consists of 75 participants. Genotypes of VDR gene in intron 8 (A/G) with BsmI restriction enzyme were determined using the PCR-RFLP method. There was no statistical difference in genotype of AA, GA and GG between PCOS women and control women (p value >0.05). Our study suggests that there was no significant association of BsmI genotypes with both obese and non obese PCOS women.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) affects 5-10% of the women in reproductive age group; however, the incidence might increase due to nutritional changes (Allahbadia and Merchant, 2008). An increase in the prevalence of PCOS among Indians is also a great concern. Observation of South Indian gynecologists from their experience reported 25-30% of the women visiting them do suffer from PCOS (Muralidhara *et al.,* 2015; Nidhi e*t al*., 2011). Clinical manifestations of PCOS include oligomenorrhea, hirsutism, alopecia, obesity, and metabolic disturbances. Further, PCOS is associated with future complications such as cardiovascular abnormalities, type 2 diabetes mellitus, dyslipidemia, risk of malignancies and infertility (Kumar *et al*., 2014).

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The aetiopathology of PCOS reported the association of many environmental as well as genetic factors in different ethnic groups (Prapas *et al.,* 2009; Fratantonio *et al.,* 2005; Diamanti *et al.,* 2006). A number of candidate genes involved in steroidogenesis (Gaaseenbeek *et al.,*2004; Qin *et al.,*2006), insulin signaling pathway (Jin *et al.,*2006; Lee *et al.,*2008) and gonadotropin secretion (Li *et al.,*2011) have been investigated to be associated with increased susceptibility to PCOS, but none is reported to have strong correlation with susceptibility to the disease. Increasing evidences suggests the contribution of vitamin D role in the pathogensis of polycystic ovary syndrome. Vitamin D deficiency might be a causal factor in the pathogenesis of insulin resistance and the metabolic syndrome in PCOS (Hahn *et al*., 2006; Wehr *et al.,* 2009). Hence vitamin D receptor (VDR) locus variations seem to have important impact on pathogenesis and insulin resistance in PCOS women. Mahmoudi in 2009 reported that "bb" genotype (presence of restriction sites for ApaI and BsmI) has

been associated with higher levels of insulin and insulin resistance in comparison to ''Ff/ff'' and "BB and Bb" genotypes (Mahmoudi, 2009). Later in 2011, Ranjzad *et al*., reported that there was significant association between VDR BsmI GG genotype and decreased levels of sex hormone binding globulin (SHBG) in PCOS women (Ranjzad *et al.,* 2011). Present investigation is the first to study the role of the VDR gene polymorphism (BsmI) in genetic susceptibility to PCOS in Indian women.

MATERIALS AND METHODS

The present study was conducted at Narayana Medical College and Hospital, Nellore, Andhra Pradesh, India during the period of October 2012 to December 2014. The study population included 75 obese and 75 non obese women with PCOS (cases) diagnosed based on Rotterdam criteria and 75 healthy women to serve as controls. All the control women had normal thyroid function, regular menstrual cycles, and no clinical signs of hyperandrogenism. All cases and controls were genetically unrelated. Diagnosis of PCOS was made on the basis of the Rotterdam criteria (Rotterdam ESHRE/ASRM consensus, 2004). Two out of three of the following are required for diagnosis: oligo- and/or anovulation (defined by the presence of oligomenorrhea or amenorrhea); clinical and/or biochemical signs of hyperandrogenism [defined by presence of hirsutism (Ferriman–Gallwey score ≥6), acne or alopecia, and/or elevated androgen levels] and polycystic ovaries by gynecological ultrasound. Patients with congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, known hypothyroidism on treatment, other confounding factors as well as individuals who are already on treatment were excluded from the study. In addition, all subjects had polycystic ovaries by ultrasonography, but this was not a required inclusion criterion.

The study was approved by Institutional Ethics Committee, Narayana Medical College and Hospital and informed consent was obtained from all the subjects. Body mass index (BMI) was defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). Obesity was defined as a BMI ≥25.10 kg/m^2 and non-obese as BMI < 25 kg/m^2. This was based on the consensus statement that the cut offs for overweight and obesity Asian Indians (Misra *et al.,* 2009). About 5 ml of venous blood was obtained during a spontaneous bleeding episode of menstrual cycle after an overnight fast and collected in plain, fluoride and EDTA tubes from all the subjects. Specimens were immediately centrifuged and serum was separated and stored at -20^oC until analysis. In both obese and non obese women with PCOS serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), glucose, insulin, total calcium, and 25-OH vitamin D were measured. Serum glucose and calcium were assessed on Huma star-600 fully automated biochemistry analyzer by using commercial kits. Serum fasting insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH) were measured with chemiluminiscence immunoassay (CLIA) method using Beckman Coulter Access – 2 fully automated analyzer. The hormone kits used in the Beckman Coulter Access analyzer 2(USA) were manufactured from Beckman Coulter, Ireland. Serum 25-OH vitamin D was estimated by high performance liquid chromatography (HPLC) with commercial column and reagents from RECIPE (Germany) and Younglin HPLC (Korea). Insulin resistance was estimated by formula for the homeostatic model assessment-insulin resistance (HOMA- IR) (Legro *et al.,* 2004). Whole blood collected in EDTA tubes was used for DNA isolation. Genomic DNA was isolated from peripheral blood (200µl) leukocytes with the commercial kit (Bioserve DNA extraction kit) according to manufacturer's instructions. Concentration of DNA was estimated using nano drop (Thermo Fischer Scientific) in ng/µl.

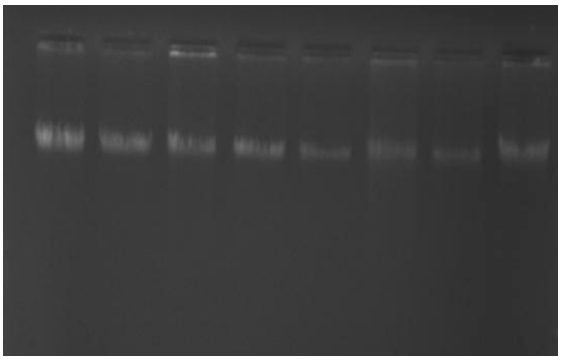

Figure 1. Genomic DNA extracted from different subjects

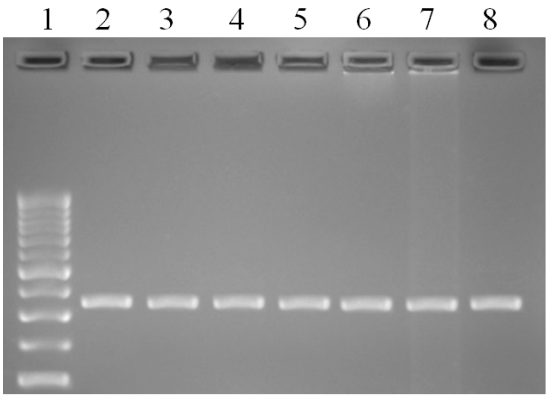

Figure 2. PCR amplification of human genome using primers targeted against VDR gene. Lane1, 100 bp DNA ladder; lane 2-8, amplified products from different genomic DNA samples

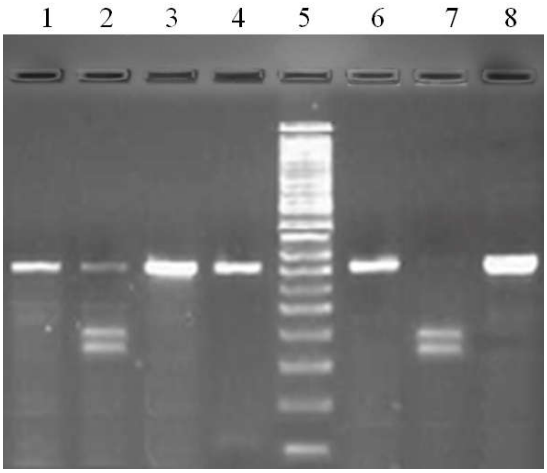

Figure 3. Restriction pattern of VDR gene fragment with BsmI. Lane 1, 3, 4, 6, 8 represents AA genotype, Lane 2 - GA genotype, Lane 7 - GG genotype and Lane 5 - 50bp DNA ladder

The genotyping of VDR gene (rs1544410) loci in intron 8 (G/A) detected by restriction enzyme BsmI using restriction fragment length polymorphism - polymerase chain reaction (RFLP-PCR) method. Polymerase chain reaction is an *in vitro* method for replicating the desired DNA fragment so that its amount increases exponentially. Figure 1 presents isolated genomic DNAs from different patients or control subjects on 1.5% agarose gel, which shows a characteristic band of genomic DNA near the well side. The genomic DNA from different subjects was amplified by polymerase chain reaction using primers targeted against the human VDR gene containing intron 8. PCR was performed using the following primers: forward: 5' GGG AGA CGT AGC AAA AGG 3' and reverse 5' AGA GGT CAA GGG TCA CTG 3'. PCR amplification of the human genome using primers of the VDR gene, as mentioned above, yielded a fragment of 360bp. Figure 2 shows a representative gel photograph of the amplified DNA product on 2% agarose gel.

Following DNA amplification, PCR products were digested with *Bsm*I (CutSmart, New England BioLabs) at 65^{o}C for 15 min (Table 1). The digested products were analyzed for the presence or absence of recognition sites by ethidium bromide staining of fragments separated through a 2% agarose gel and photographed. Three types of genotypes have been observed in the study subjects represented as AA, AG and GG. The genotype designated as AA shows absence of restriction site and yielded only one band in agarose gel electrophoresis at 360bp region which is of same size of PCR product. The genotype designated by GG shows presence of restriction site and yielded two bands of size 191 bp and 169 bp. The GA genotype presented with three bands in gel electrophoresis and size of fragments is 360 bp, 191bp, 169 bp (Figure 3).

Statistical analysis

Hardy Weinberg Equilibrium was tested to find out the gene polymorphism. The chi-square (χ^2) test was performed to compare VDR BsmI (rs1544410) intron 8 (G/A) genotypic and allelic distributions between patients and healthy control group. The association between genotypes was examined by odds ratio with 95% Confidence Interval (CI). Chi-Square analysis using Open epi 6 software (open Epi version 2.3.1 from the department of epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). Allelic frequencies were calculated according to the number of different alleles observed and total number of alleles examined.

RESULTS

The studied group consisted of 75 obese PCOS women, 75 non obese PCOS women and 75 healthy women as normal controls with age ranging from 19 to 36 years. The mean age of obese PCS women was, 25.96 ± 3.22 years, non obese PCOS women was 25.60 ± 4.08, and that of control women was 26.06 ± 3.58. Statistically significant difference between cases and controls was not found regarding age (p-value >0.05). But with respect to BMI (kg/m^2) there is statistically significant difference between obese PCOS women (mean ± SD, 28.29 ± 3.20), non obese PCOS women (mean ± SD, 21.91 ± 1.30) and controls (mean ± SD, 24.86 ± 2.98) (p <0.001). PCOS women had a higher LH, FSH levels and low vitamin D and calcium levels when compared to controls. The LH/FSH ratio was significantly higher in obese PCOS women (1.02 ± 0.15) (p <0.05) than non obese PCOS women (0.95 ± 0.15) when compared to healthy controls (0.94 ± 0.13) (Table 2).

Table 1. Type of SNPs, site of SNPs, and PCR Conditions

Gene	Location	PCR details
VDR/BsmI (rs1544410)	Intron VIII (G/A)	PCR protocol: 94^{o}C for 1 min, 54^{o}C for 1 min, 72^{o}C for 1 min for 30 cycles. 5' GGG AGA CGT AGC AAA AGG 3' 5' AGA GGT CAA GGG TCA CTG 3' un-cut PCR product size: 360(bp) Restriction enzymes, Incubation temperature: Bsml, 65°C for 15 min

Table 2 Demographic and clinical characteristics of control and PCOS women

Parameter	Control women	Obese PCOS women	Non obese PCOS women
Age (Years)	26.06 ± 3.58	25.96 ± 3.22	25.60 ± 4.08
BMI (kg/m^2)	24.86±2.9	27.82 ± 3.14**	21.91±1.30**
WC (cm)	71.81±4.96	85.55±5.40**	74.53±5.31**
HC(cm)	86.29±5.87	87.83±4.22	83.20±11.16*
W/H ratio	0.83±0.07	0.96±0.05**	0.88±0.05**
Glucose (mg/dl)	86.69±7.60	83.48±5.48**	81.89±4.90**
Insulin (µIU/ml)	6.65±1.25	7.29±1.29**	5.93±1.12**
HOMA – IR	1.42± 0.36	1.49±0.31	1.20±0.27
LH (mIU/L)	6.00±1.76	8.05±1.24**	6.78±2.05**
FSH (mIU/L)	6.28±1.55	7.85±1.02**	7.09±1.89**
LH/FSH	0.94 ± 0.13	1.02 ± 0.15**	0.95 ± 0.15
Vitamin D (ng/ml)	31.91±8.28	30.36±8.33	30.99±6.64
Ca (mg/dl)	8.55±0.50	7.36±1.08**	6.93±1.17**
Phosphorous (mg/dl)	4.41±0.75	4.18±0.31*	4.36±0.33

*Significant at 0.05 level with control group; **Significant at 0.01 level with control group

Table 3. Distribution of VDR gene intron 8 (G/A) genotypes and allelic frequencies of the obese POCS women and control women

Study group of VDR genotype					Allele frequency		
	AA	GA	GG	TOTAL	A	G	TOTAL
Obese PCOS (n %)	50	20	5	75	120 (0.8)	30 (0.2)	150
Control women (n %)	62	12	1	75	136 (0.91)	14 (0.09)	150

For GG vs. AA; χ^2 = 3.39; p = 0.07; odds ratio = 0.16 (95% CI: 0.018 – 1.42); for GG vs. GA + AA - χ^2 = 2.7; p = 0.09; odds ratio = 5.2 (95% CI: 0.6 – 46.37); G vs A χ^2 = 6.79; p=0.009; odds ratio = 0.41 (95% CI: 0.2-0.81)

Table 4. Distribution of VDR gene intron 8 (G/A) genotypes and allelic frequencies of the non obese POCS women and control women

Study group of VDR genotype					Allele frequency		
	AA	GA	GG	TOTAL	A	G	TOTAL
Non obese PCOS (n %)	47	24	4	75	118(0.78)	32 (0.22)	150
Control women (n %)	62	12	1	75	136 (0.91)	14 (0.09)	150

For GG vs. AA; $\chi^2 = 2.6$; p = 0.1; odds ratio = 0.19 (95% CI: 0.02 – 1.7); for GG vs. GA + AA - $\chi^2 = 1.85$; p = 0.17; odds ratio = 4.16 (95% CI: 0.45 – 38.2); G vs A; $\chi^2 = 8.29$, p=0.003: Odds ratio = 0.37 (95% CI: 0.19-0.74)

The genotypic distribution of VDR BsmI (rs1544410) intron 8 (A/G) polymorphism and allelic frequency of A and G alleles in obese and non obese PCOS women and controls have been given in Table 2 and Table 3. In the case of VDR BsmI (rs1544410) intron 8 (A/G), obese PCOS women ($\chi^2 = 3.39 < 3.84$, p value with degree of freedom 2 = 0.07 >0.05) and non obese PCOS women ($\chi^2 = 2.6 < 3.84$, p value with degree of freedom 2 = 0.1 >0.05) were consistent with Hardy Weinberg equilibrium. VDR BsmI (rs1544410) intron 8 (A/G) AA, GA, GG, A and G genotypic/allelic frequencies were 50 (66.6%), 20 (26.7%), 5(6.7%), 120(80%), and 30(20%) in obese PCOS women, 47(62.7%), 24(32%), 4(5.3%), 118(78.6%), and 32(21.3%) in non obese PCOS women and 62(82.6%), 12(16%), 1(1.4%), 136(90.6%), and 14(9.4%) in controls, respectively. The frequencies of A and G alleles were 0.8% and 0.2% in obese PCOS women and that of non obese PCOS women were 0.78%, 0.22%. While the frequencies of A and G alleles were 0.91% and 0.09% in controls. Statistical analysis showed that the differences in genotypic frequencies between the cases and controls were not statistically significant regarding VDR BsmI (rs1544410) intron 8 (A>G) (p >0.05) (Table 3 and Table 4). Our study reported that there was no statistical difference in genotype of AA, GA and GG between obese PCOS/non obese PCOS women and control women (p value >0.05). But, we observed statistically significant difference in allelic frequencies of A and G in both obese and non obese PCOS women when compared to controls (p <0.05).

DISCUSSION

The aim of present study was to assess the role of VDR BsmI (rs1544410) (A/G) genetic variation in PCOS for the first time in Indian women. Current understanding of the molecular actions of vitamin D in the fertility of women and calcium homeostasis prompted the design of the present study. The goal of this investigation was to study whether the VDR BSmI gene variant in intron 8 (A/G) (rs1544410) is related to onset of PCOS for the first time in Indian women. PCOS is known as a syndrome and affects ovarian function. PCOS most commonly occur during adolescence and characterized by several different features including amenorrhoea, oligomenorrhoea, infertility as well as other metabolic problems in medical findings (Golbahar *et al.,* 2012). It has been indicated that some females with syndrome will show polycystic ovary without clinical features of androgen excess. A patient with PCOS has an increased risk of obesity, bleeding disorders, hyperandrogenemia, endometrial carcinoma, breast cancer, chronic anovulation, infertility, insulin resistance, diabetes, hypertension, primary hyperparathyroidism, dyslipidemia and coronary artery disease (Pfeifer, 2005).

Research studies indicated that the reason of the ovarian overproduction of testosterone in PCOS women is due by inability of women to mediate insulin effectively (insulin resistance or hyperinsulinemia) (Mahmoudi, 2009; Wehr *et al.,* 2011). Hyperandrogenemia and insulin resistance are important indicators of PCOS. In this condition, level of insulin hormone within the blood is too high; therefore the ovaries produce higher level of testosterone (Wehr *et al.,* 2011). SHBG is a carrier protein which regulates the level of unbound steroids in peripheral blood (Golbahar *et al.,* 2012). It has been demonstrated that VDR genetic variations have been associated with LH and SHBG levels in PCOS women (Ranjzad *et al.,* 2011). It has been demonstrated that SHBG expression is reduced in the stromal compartment of endometria of women with polycystic ovary syndrome (Maliqueo *et al.,* 2007). Increase in the levels of androgens bioavailability result in hyperandrogenemia by hyperinsulinemia in PCOS women with VDR BsmI GG genotypes via lower serum level of SHBG (Ranjzad *et al.,* 2011; Mahmoudi, 2009).

Insulin resistance is in association with reproductive abnormalities in PCOS women. Insulin resistance is correlated with vitamin D metabolism in PCOS (Pfeifer 2005). Biological responses and functions of vitamin D are mediated via the VDR within the vitamin D endocrine system in more than 30 target tissues (Chiu *et al*., 2001; Harris *et al*., 1997; Vigo Gago *et al*., 2005; Holick 2007; Kinuta *et al*., 2000). In the body, vitamin D regulates calcium homeostasis; an important function in development of the skeletal system as well as in bone mineralization (Hassan *et al*., 2012). Since vitamin D is the main regulator for calcium and phosphate translocation.

From the small intestine into the circulation, defects observed in the mutant VDR and calcium absorption lead to decreased level of mineral transport and hypocalcemia (Hassan *et al*., 2012; Ranjzad *et al.,* 2011). Vitamin D and calcium repletion predict reproductive success following fertilization (Brannon and Picciano, 2011; Grundmann and Von Versen-Hoynck, 2011). Liang *et al*., indicated a dynamic role for Ca level in oocyte maturation and early embryonic development. Other studies are consistent with Liang *et al*., and imply that regulation of the ovum activation, follicular development and mammalian embryo development are calcium dependent (Liang *et al*., 2011). The findings of Oh and Barrett-Connor in 2002, suggest that VDR gene variant may be associated with glucose intolerance independent of defective insulin secretion and with insulin resistance (Oh and Barrett-Connor, 2002). Mahmoudi, indicated that VDR gene variant may affect PCOS development as well as insulin resistance in women with PCOS. Insulin resistance and increased levels of LH are usual signs of PCOS. Higher levels of LH, not only has an effect on oocyte maturity and human reproduction but also on lower

fertility and higher miscarriage prevalence. Still there were controversial findings about the action of LH on oocyte, embryo quality, fertility, implantation and miscarriage prevalence (Oh and Barrett-Connor, 2002; Gordon *et al*., 2001). In our study though PCOS women carrying the VDR BsmI "GG" and "GA" genotype showed low vitamin D, calcium, and phosphorous levels as compared with individuals in the "AA" genotype, but it is not statistically significant. The molecular mechanism through which this polymorphism influences LH levels is not known at present; however, previous studies have shown significant associations between VDR gene variants and insulin resistance on one side (Oh and Barrett-Connor, 2002), and insulin resistance and LH on the other. Furthermore, it has been suggested a modulating role of 1, 25 – OH vitamin D in the control of FSH secretion. Therefore, these findings are consistent with a recent report (Mahmoudi, 2009) that showed the VDR gene variants might have a role in pathogenesis of PCOS.

In the present study out of 75 obese POCS women, 50 women had AA genotype, 20 women had GA and 5 women showed GG genotype. In non obese PCOS women group 47 women presented AA genotype, 24 women had GA genotype, and 4 women presented with GG genotype. While in control 62 women were presented with AA genotype, 12 women had GA and only one women presented with GG genotype. In our study, statistical analysis showed that the differences in genotypic/allelic frequencies between the cases (obese and non obese PCOS women) and controls were not statistically significant with respect to VDR BsmI (rs1544410) intron 8 (A>G) ($p > 0.05$). The findings of the present study were consistent with some reports (Wehr *et al*., 2011; Bagheri *et al*., 2012) and inconsistent with other (Mahmoudi, 2009; Ranjzad *et al*., 2011; Jain *et al*., 2012) regarding the VDR BsmI gene variant. The exact aetiopathogenesis of PCOS are not known regarding vitamin D and insulin resistance. Several molecular mechanisms have been suggested to describe the relationship between the VDR locus variations and PCOS in different ethnic groups. We had some limitations regarding low sample size. Studies with a large sample size and more information such as other candidate gene variants, haplotypes and genetic linkage assessment are needed for further analysis (Tabor *et al*., 2002; Cardon and Palmer, 2003; Colhoun *et al*., 2003).

Conclusion

Although our sample size is small, this study was well designed and focused on the role of vitamin D metabolism related gene polymorphism on metabolic and biochemical characteristics of Indian women with PCOS. It can be concluded that VDR BsmI (rs1544410) intron 8 (A/G) were not associated with PCOS susceptibility in our population. However, further studies with increased numbers of PCOS patients are required to validate these findings.

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Conflict of interest

There was no conflict of interest in this study.

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