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GENETIC POLYMORPHISMS OF VITAMIN D RECEPTOR IN POLYCYSTIC OVARIAN SYNDROME: A CASE CONTROL STUDY

Soubia Mallick, Mehir un Nisa Iqbal and Taseer Ahmed Khan*

Department of Physiology, University of Karachi, Karachi-75270. Pakistan
*Corresponding author: Email: takhan@uok.edu.pk

ABSTRACT

Vitamin D receptor (VDR) gene polymorphisms might play a role in the development of PCOS by exerting effects on LH, testosterone and sex hormone binding globulin (SHBG) concentration. VDR gene is highly polymorphic and several VDR polymorphisms have been recognized but the most commonly studied VDR variants are Fok1, Taq1 and Apa1. Therefore, present study was first of its kind to address the association of VDR gene polymorphism in PCOS among Pakistani females. It is a case control study consists of 150 women diagnosed with PCOS on the basis of Rotterdam criteria and 100 healthy participants. Genomic DNA was extracted through salting out method and VDR genotyping was done by PCR-RFLP analysis. Statistical analysis was conducted by SPSS (version 22) considering $p<0.05$. Hardy-Weinberg equilibrium (HWE) for controls was calculated. Pearson chi-square (χ^2) along with unadjusted odds ratio (OR) and 95% confidence intervals (95% CI) was done to evaluate the association and risk respectively among cases and controls. The study revealed that Fok1 ff (OR=11.803, 95%CI=1.488–93.62) genotype was significantly associated with the risk of developing PCOS. The study concluded that VDR gene polymorphisms may be associated with PCOS.

Keywords: Vitamin D receptor, genetic polymorphism, polycystic ovarian syndrome, PCR, RFLP.

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a major metabolic, gynaecological endocrine disorder which affects females of reproductive age (12-45 years). It is characterized by menstrual irregularity, hyperandrogenism, polycystic ovaries, insulin resistance (IR) and obesity. The prevalence rate of PCOS is around 5-10% (Allahbadia and Merchant, 2008) worldwide however a controversy exists in Pakistan over its prevalence in the country with few reports determining the prevalence rate of 40.9% among infertile female. The hormonal manifestations of PCOS include high levels of androgen, luteinizing hormone (LH) with normal follicle stimulating hormone (FSH). The metabolic changes include insulin resistance, hyperinsulinemia and abdominal obesity (Mahmoudi, 2009) which may lead to the development of non-insulin dependent diabetes mellitus (NIDDM). PCOS is a multigenic disorder but the contributions of gene for the pathogenesis of PCOS have not been clearly understood. However, studies have documented that vitamin D receptor gene polymorphisms may play an important role in the development of PCOS (El-Shal *et al.*, 2013).

Vitamin D, a secosteroid, regulates learning activities, bone metabolism, cell proliferation, differentiation, immune responses, angiogenesis, prevention of diabetes and cancers. However, the deficiency of vitamin D may be involved in the development of various widespread diseases including PCOS (Feldman *et al.*, 2014). Vitamin D3 facilitates its action upon binding to its vitamin D receptor (VDR), intra-nuclear receptor transverses its signal transduction by connecting with VDR response element (VDRE) of target gene. The gene for the VDR is situated at the chromosome no. 12q13-14. The polymorphisms in VDR gene might play a role in the development of PCOS by exerting effects on LH, testosterone and sex hormone binding globulin (SHBG) concentration (Ranjzad *et al.*, 2011). VDR gene is highly polymorphic since several VDR polymorphisms have been recognized but the most commonly studied VDR variants are Fok1, Taq1 and Apa1. Fok1 polymorphism is only documented polymorphism of that causes generation of altered protein (Arai *et al.*, 1997) whereas other SNPs including Bsm1, Apa1 and Taq1 are involved in the VDR mRNA stability (Durrin *et al.*, 1999).

Many studies have documented that VDR polymorphism is associated with the development of phenotypes of PCOS. Polymorphism of VDR genes (Fok1, Apa1 and Taq1) may be involved in the etiology of metabolic abnormalities, regulation of endocrine parameters and adiposity phenotypes (Ochs-Balcom *et al.*, 2011). VDR is present in pancreatic beta cells which reveal the fact that vitamin D affected the secretion of insulin thus deficiency of vitamin D or VDR polymorphism may results in the hyperinsulinemia in PCOS. Apa1 and Taq1 genes are significantly linked with overweight and obesity (Vasilopoulos *et al.*, 2013) whereas Fok1 genotype is associated with an increase in waist-to-hip ratio (WHR) (Sweeney *et al.*, 2005). However, the Taq1 polymorphism (TT

genotype) were reported to cause a ~30% increase in the incidence of obesity (Tworowska-Bardzinska *et al.*, 2008). Moreover, Apa1 (CC) genotype is related with the higher risk of having PCOS (Mahmoudi, 2009). In the pathophysiology of PCOS, androgen plays an important role by causing an ovulation and polycystic ovarian morphology. It also contributes to insulin resistance and abdominal adiposity which leads to severity and progression of the disease (Maestro *et al.*, 2003).

PCOS has never been studied in Pakistan with its genetic perspective and much has been focused on either hormone profile or drug interventional studies. Therefore, present investigation is the first of its kind on Pakistani population with an attempt to associate VDR polymorphisms (Fok1, Apa1 and Taq1) and risk of PCOS since no reported literature is available up to date on Pakistani population which correlates the role of vitamin D receptor gene polymorphism and pathogenesis of PCOS. In Asian countries particularly Pakistan, where vitamin D deficiency is common (Masood *et al.*, 2010), supplementation of vitamin D is practiced to normalize various metabolic processes, however, the outcomes are not promising. In fact, a mechanism may be impaired in the pathway of vitamin D or there may be an involvement of genetic polymorphisms in the VDR which may be a cause of developing PCOS which is the main insight in planning this study. Thus, the findings of this study will provide a better understanding for the treatment of PCOS since VDR polymorphisms along with vitamin D levels should be considered during disease therapy.

MATERIALS AND METHODS

Study participants

This is a retrospective case control study with a total of 250 female subjects (150 cases and 100 controls) aged between 12-45 years. Cases were diagnosed with PCOS on the basis of Rotterdam criterion and were recruited from two tertiary hospitals in Karachi over a period from January 2013 to March 2014, whereas, un-match controls were healthy eumenorrheic, non-hirsute females without PCOS and with no clinical evidence of any other endocrine and non-endocrine abnormality. A thorough investigation about family history of PCOS, menstrual history, lifestyle and dietary patterns, Body Mass Index (BMI), Waist to hip ratio (WHR) and other parameters was done through detailed self-structured questionnaire. The study was approved by the Board of Advanced Studies and Research (Approval#10(S) 09 24042013) with ethical standards according to the Helsinki declaration. Written informed consent for participation in the study was obtained from participants.

Genomic DNA extraction

Blood samples were collected in EDTA tubes from all study participants through antecubital vein. Genomic DNA was isolated from peripheral lymphocytes through 'proteinase K' method using whole blood. In brief, the 500µL of whole blood was washed three times with 1000µL of haemolytic solution followed by digestion in the presence of proteinase-K (20µL) and 10% sodium dodecyl sulphate (40µL) at 56°C for 30 min. After digestion proteins were precipitated using 250µL of 6M NaCl. Consequently DNA was extracted from supernatant using isopropanol followed by washing with 70% ethanol. The extracted DNA was resuspended in nuclease free water and stored at -86°C till further use.

Genetic analysis of VDR Fok1 (rs2228570), Apa1 (rs7975232) and Taq1 (rs731236) polymorphisms

Polymorphic sites in VDR genes (Fok1, Apa1 and Taq1) among PCOS predisposed subjects were determined using PCR-RFLP method. Genomic DNA was amplified through master mix (Kapa Biosystems, USA) in a reaction volume of 25µL on an automated thermal cycler (Veriti TM, Applied BioSystems, USA) using primers as outlined in Table I.

The PCR conditions for all reactions include: initial denaturation at 94°C for 10mins followed by 40 cycles of denaturation at 94°C for 30sec, annealing (59°C for Fok1 and 62°C for Apa1 and Taq1) for 10 sec, extension at 72°C for 30sec with a final extension of 5min at 72°C. The PCR products were digested using Fok1 (37°C), Apa1 (37°C) and Taq1 (65°C) restriction endonucleases (Thermo scientific USA). All the pre and post digested products were run on 1.5% agarose gel added with 0.5 µg/ml ethidium bromide against a 100bp ladder and visualized using gel documentation system ChemiDoc-It2 (UVP, UK) through Vision works LS software (version 7.1). The sizes of all genotypes are given in Table I. Two independent persons, who were blinded to the cases and controls, were scored the genotypes status. Overall call rates for the Bsm1, Fok1 and Taq1 SNPs were 96.8, 97.0 and 97.9%, respectively.

Table 1. PCR-RFLP pattern of Fok1, Apa1 and Taq1 polymorphisms of vitamin D receptor gene.

Polymorphisms	Primers	PCR product size (bp)	RFLP
Fok1			
rs2228570	F=5'- AGCTGGCCCTGGCACTGACTCTGCTCT-3'	265	FF= 265
	R=5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'		ff=196, 69
Taq1			TT=495, 245
rs731236			tt=290, 245, 205
	ATF: 5'-CAGAGCATGGACAGGGAGCAA-3'	740	
	ATR: 5'-CACTTCGAGCACAAGGGGCGTTAGC-3'		
Apa1			AA=740
rs7975232			aa=530, 210

Statistical analysis:

All data was subjected to statistical analysis using IBM SPSS (version 22). Chi square (χ^2) goodness of fit was estimated to determine if the observed genotype frequencies of controls deviated from Hardy-Weinberg equilibrium (HWE). Pearson chi-square (χ^2) was done to find out the statistical significance differences in the frequencies of genetic variants among both groups (cases and controls) association between PCOS and healthy volunteers with respect to specific genotypes. The unadjusted odds ratio (OR) and 95% confidence intervals (95% CI) were calculated via binary logistic regression analysis as a measure of association which determine risk however, significance level was employed for comparison at $p < 0.05$.

RESULTS

The present study is a retrospective case control study in which 150 patients and 100 unmatched healthy volunteers with the age range of 12-45yrs were enrolled. Out of 250, 186 participants (95 cases and 91 controls) successfully participated in the study and remaining 64 were not eligible for the study because they had endocrine related and other abnormalities. Out of 186, 160 samples were amplified positively and amplified samples were digested via RFLP.

The data is stratified on the basis of BMIs, WHR, menarcheal age, hirsutism, acne and menstrual cycle irregularity among PCOS patients and healthy participants are depicted in Table 2.

The genetic distribution of all polymorphisms (Fok1, Apa1, and Taq1) in healthy participants was in correspondence with HWE (Table 3). The association between VDR gene polymorphism and PCOS risk among cases and controls is shown in Table 3. A statistical significant difference in genotype frequencies among cases and controls was observed for Fok1 polymorphism. Moreover, the 'ff' genotype was found to be at risk which is highly statistically significant. The genotype distribution of Apa1 was significantly associated with PCOS, however, risk of PCOS with Apa1 polymorphism was not obtained. Furthermore, no statistically significant difference was obtained in the genotype distribution of Taq1 but TT and Tt genotype was found to be at risk.

DISCUSSION

The PCOS is a heterogeneous group of disorder affecting 5-10% (Allahbadia and Merchant, 2008) females of reproductive age globally and classically characterized by the ovarian dysfunction, heperinsulinemia, hyperandrogenism and cyst and stromal formation in ovarian tissues. The prognosis of ovarian tissues in Pakistani female is still uncertain, which is the major impediment about the prevalence of PCOS among Pakistani women. Cystic ovaries in general and PCO in specific are major health issues in Pakistan leading towards infertility. Even though, PCOS pathogenesis remains debatable its manifestation becomes possible only at the time of puberty (Franks, 2008). Since PCOS is a multigenic disorder association of multiple genes including VDR and its

polymorphisms in the onset of PCOS have been explored. Vitamin D3 is reported to have an inverse association with PCOS. In addition, vitamin D also causes transcription of numerous genes via VDR which is involved in the regulation of endocrine function (Parikh *et al.*, 2010), ovarian folliculogenesis (Sun *et al.*, 2010), and pancreatic beta cells (Bikle, 2009). More than 200 SNPs in VDR gene have been identified. However, most commonly associated SNPs with the PCOS are Fok1, Taq1, Bsm1 and Apa1 (Jedrzejuk *et al.*, 2015). To date no data is available regarding the association of VDR polymorphism and risk of PCOS in Pakistani population. Therefore this study examines the VDR polymorphisms association and risk of PCOS in Pakistani women.

Table 2. Clinical characteristics of study participants and their association with PCOS.

Factors	Cases (N)	Controls(N)	$\chi 2$	P-value	*OR (95% CI)	P-value
BMI						
Normal (19-23)	18	30			1	
Obese (>25)	64	12	78.93	0.000	8.889 (3.801-20.786)	0.000
Overweight (23-25)	12	07			2.857 (0.951-8.585)	0.061
Underweight (<19)	01	42			0.040 (0.005-0.315)	0.002
WHR						
Acceptable	16	47	25.139	0.000	1	
Unacceptable	79	44			5.274 (2.681-10.375)	0.000
Menarcheal Age						
Ideal (12-14)	09	81			1	
Late (15-20)	12	07	1.244E2	0.000	15.429 (4.842-49.164)	0.000
Early (9-11)	74	03			222.00 (57.89-851.32)	0.000
Hirsutism						
No	32	77	49.697	0.000	1	
Yes	63	14			10.828 (5.319-22.034)	0.000
Acne						
No	41	59	8.786	0.003	1	
Yes	54	32			2.428 (1.344-4.388)	0.003
Menstrual Cycle Per Year						
Normal (10-17 cycles/year)	27	85			1	
Polymenorrhea (18-20 cycles/year)	03	02			4.722 (0.749-29.759)	0.098
Oligomenorrhea (4-9 cycles/year)	52	04	84.332	0.000	40.926 (13.55-123.60)	0.000
Amenorrhea (0-3 cycle(s)/year)	13	00			5.086E9 (0.000-NC**)	0.998

*Odds ratio along with 95%CI were calculated using binary logistic regression analysis; ** NC= Not calculated

Among the most dominant and common pathogenic features of PCOS is obesity which can be determined through BMI, and WHR. This study revealed that obesity was associated with the risk of developing PCOS which is in accordance with previous studies (El-Shal *et al.*, 2013). However, a study carried out on Iraqi infertile women who showed a non-significant difference in BMI among cases and controls (Bayan and Alalaf, 2013). Present investigation also revealed that high WHR has a positive association with the PCOS risk as reported earlier by Bayan and Alalaf (Bayan and Alalaf, 2013) contrary finding was observed in a study at Greek island (Diamanti-Kandarakis *et al.*, 1999). Hirsutism and acne is most important characteristic feature of PCOS and it was observed that hirsute subject and those having acne problems were at risk of developing PCOS which is in agreement with the previous study on East Asian and Pacific Islander women (Williamson *et al.*, 2001) whereas Asian women in comparison to Mediterranean or Middle Eastern are less prone to develop hirsutism (Wijeyaratne *et al.*, 2002). Previous studies have reported a rate of acne is 12% - 14% (Azziz *et al.*, 2005), whereas, its prevalence is 48.7% in Italian women (Belosi *et al.*, 2006) with PCOS. The findings of present study revealed that menstrual irregularity is highly associated with the PCOS among cases and controls which was in accordance to previous studies (Bayan and Alalaf, 2013).

Table 3. Association of VDR polymorphisms and PCOS risk.

Polymorphisms	Genotype	Genotype frequency (N)		HWE ($\chi 2$)	P-value	Pearson's $\chi 2$	P-value	OR (95 % CI)
		Cases	Controls					
Fok1	FF	61	60	0.218	0.64	16.832	0	1
	Ff	6	20					0.295 (0.111 – 0.786)
	ff	12	1					11.803 (1.488 – 93.62)
	Total	79	81					
Apa1	AA	25	20	0.832	0.361	1.29E+02	0	1
	Aa	26	34					0.612 (0.281- 1.333)
	aa	14	22					0.509 (0.209- 1.242)
	Total	65	76					
Taq1	tt	8	12	2.535	0.111	0.588	0.745	1
	Tt	27	27					1.5 (0.529- 4.250)
	TT	30	34					1.324 (0.477- 3.672)
	Total	65	73					

*Odds ratio along with 95%CI were calculated using binary logistic regression analysis

A significant association between Fok1 polymorphism and PCOS among Pakistani females was observed in present investigation which is in accordance with Sweeney *et al.,* (2005). However, a non-significant association between Fok1 polymorphism and PCOS risk was described by Jedrzejuk *et al.,* (2015). The results also suggested a positive association between Apa1 polymorphism and PCOS which is similar to the study of Mahmoudi, (2009) whereas opposite results were reported by Jedrzejuk *et al.,(*2015). This investigation also revealed that a statistically non-significant difference was found between Taq1 polymorphism and PCOS which is in agreement with Jedrzejuk *et al.,* (2015) and contrary with Ranjzad *et al.,* (2011). In addition, the odd ratios described Fok1 ff genotype and Taq1 TT and tt genotype are at PCOS risk. Studies have documented that Fok1 (Sweeney *et al.,* 2005) and Taq1 polymorphisms (Ranjzad *et al.,* 2011) were found to be a risk factor for PCOS.

Many researchers time to time addressed on the common issues of PCOS including high serum androgen levels, IR, obesity, menstrual abnormalities and inflammation but these pathways have not promisingly resolve the mystery of PCOS. However, deficiency of vitamin D may provide the possible mechanisms behind the etiology of above mentioned issues of PCOS.

VDR is expressed on the preadipocytes which reveal that it inhibits the differentiation and stimulate the synthesis as well as secretion of lipoprotein lipase. The VDR polymorphisms lead to the development of obesity by increasing the cellular differentiation and inhibiting the lipoprotein lipase in PCOS women.

Vitamin D plays an important role in the regulation of beta cell functions of pancreas i.e. secretion and actions of insulin. Vitamin D acts on the pancreatic beta cells to enhance the calcium influx which causes increase insulin secretion. In addition, vitamin D/VDR complex enhances insulin actions by increasing the expression of cell surface insulin receptors and increases insulin sensitivity thereby increasing the transportation of glucose within cells in response to insulin. So in case of hypovitaminosis D or VDR polymorphisms, insulin secretion and actions are altered and may develop IR. Moreover, vitamin D also acts as an immunomodulator and its deficiency may enhance the inflammatory processes which also have a role in IR (Bikle, 2009).

VDR and metabolizing enzymes of vitamin D are present in male and female reproductive tissues. A significant gonadal failure, low sperm count, its motility and abnormal histological features of testis, ovaries and uterus are present in VDR knockout mice (Lerchbaum and Obermayer-Pietsch, 2012). Gonadal steroidogenesis is also regulated by vitamin D. it stimulates progesterone, estradiol and estrone production by 13%, 9% and 21% respectively in ovarian tissues (Parikh *et al.,* 2010). Vitamin D also involved in the development and maturation of oocytes. In 1α hydroxylase knockout mice, infertility is observed due to the reduced estrogen and progesterone levels followed by increased gonadotropins levels and resulting in defective development of follicles and corpus luteum along with the hypoplasia of uterus (Sun *et al.,* 2010).

Limitations of the study

The study had the limitations of small sample size. Genetic studies involving haplotypes, other candidate gene variants, genetic linkage assessment with a large sample size are thought to be useful for further evaluation regarding relationship between VDR gene polymorphisms and PCOS.

CONCLUSION

This study revealed that VDR polymorphisms specifically Fok1 ff genotype may significantly associated with the risk of developing PCOS.

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