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Original Article

Biochemistry Section

Assessment of Vitamin D Receptor Gene Polymorphism in Iraqi Women with Polycystic Ovary Syndrome

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ABSTRACT

Introduction: Polycystic Ovary Syndrome (PCOS) is the most common hormonal disorder in women that occurs mainly in the reproductive age. It is suggested to be highly complex and heterogeneous disorder with an uncertain cause.

Genes included in vitamin D metabolism have been assumed as candidate genes for the PCOS susceptibility. Vitamin D receptor gene polymorphisms are suggested to have an influential role in insulin metabolism in women with PCOS.

Aim: To investigate the possible association between Cdx2 (G/A) Single Nucleotide Polymorphism (SNP) of vitamin D receptor gene and the risk of polycystic ovary syndrome.

Materials and Methods: The present Case-control study involved 88 women from 18 to 34 years of age in which Group 1 consisted of 45 newly diagnosed women with PCOS while Group 2 consisted of 43 women without PCOS that acted as controls.

DNA samples were amplified and analysed for the Cdx2 (G/A) SNP of vitamin D receptor gene. Genotypic frequency distribution of Cdx2 polymorphism of vitamin D receptor gene was calculated in women with PCOS and controls. From each serum sample 25-hydroxy vitamin D, calcium, LH, FSH, free testosterone, insulin, and glucose was calculated according to AA, GA, and GG genotypic distribution.

Results: There was no significant difference in genotypic distributions of Cdx2 polymorphism of vitamin D receptor gene between patients and controls. In addition, the results of patients group were found to be significantly lower for fasting serum glucose (p=0.02), insulin (p=0.01), and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) (p<0.001) inindividuals with AA genotype than individual with GA and GG genotype. While, significantly higher levels of LH (p=0.002) and LH/FSH ratio (p=0.003) inindividual with GG genotype than individual with GA and AA genotype, and no significant difference in mean value of FSH (p=0.148) and free testosterone (p=0.091) between GG, GA, and AA carriers. Likewise, the results were observed significantly lower levels for serum 25-hydroxy vitamin D in GG carriers than GA and AA carriers for both patients (p<0.001) and controls (p<0.001), with no significant difference in mean value of calcium levels between GG, GA and AA carriers for patients (p=0.949) and controls (p=0.46).

Conclusion: Cdx2 polymorphism of vitamin D receptor gene has an association with severity of clinical features in PCOS; however, not with risk of development of the disease meaning that genetic variation is not directly linked to risk of this syndrome but may indirectly affect disease development via regulation of vitamin D levels.

Keywords: Cdx2 polymorphism, Hormonal disorder, Single nucleotide polymorphism

INTRODUCTION

Polycystic Ovary Syndrome is the most common hormonal disorder in women that occurs mainly in reproductive age. It is suggested to be highly complex and heterogeneous disorder with an uncertain cause [1].

Insulin Resistance (IR) is predominant in women with this disorder which contribute to reproductive and metabolic defect seen in this syndrome [2]. IR and compensatory hyperinsulinemia lead to increased synthesis of ovarian androgen causing follicular arrest and anovulation [3]. Abnormality in folliculogenesis and steroidogenesis participate in the development of this disorder. These abnormalities may originate from both genetic susceptibility and environmental factors [4].

Genes included in vitamin D metabolism have been assumed as candidate genes for the PCOS susceptibility. Vitamin D Receptor (VDR) gene polymorphisms were suggested to have an influential role in insulin metabolism in women with PCOS [5].

Many allelic variations (polymorphisms) of the *VDR* gene located on chromosome 12 are found naturally in the human population [6], with influential differences according to race and ethnicity [7]. Approximately, 200 SNPs of the vitamin D receptor gene are found to be involved [8]. Cdx2, FokI, BsmI, ApaI, and TaqI were regarded to be the most common allelic variants in the human population [9].

It has been assumed that SNPs within the *VDR* gene could affect the VDR amount, stability, and activity as well as the transcription rate of VDR gene [10].

Vitamin D receptor gene is composed of eight exons in the coding region and six extra exons (1a-1f) in the regulatory region, which also includes several promoters [2-9]. Cdx-2 binding region is one functional SNP of the human VDR-1a promoter region [11]. Cdx2 acts as a transcription factor of VDR [12]. Cdx2 (G/A) expression leads to a *VDR* gene with a defective binding region for the intestine specific-transcription factor CDX2 [12]. This results in diminished intestinal VDR levels [13], with a decrease in active vitamin D that enhances calcium transport proteins and calcium absorption [14]. Therefore, the objective of the present study was to investigate the possible association between Cdx2 (G/A) single nucleotide polymorphism of the *VDR* gene and the risk of polycystic ovary syndrome.

MATERIALS AND METHODS

The present case-control study involved 88 women from 18 to 34 years of age. Women were selected from Infertility Center-Baghdad Teaching Hospital during the period of March 2017 to June 2017. Informed consent was taken from each participant. The study was approved by the Ethical Committee of the College of Medicine/ University of Baghdad.

Women with PCOS were diagnosed according to Rotterdam criteria [15], when two out of three following criteria are present, these include oligoovulation and/or anovulation, clinical and/or biochemical evidence of hyperandrogenism and polycystic ovaries as seen by ultrasound. In addition, controls were defined as healthy women in reproductive age with regular menstrual periods and without PCOS manifestations (hirsutism, acne, alopecia, etc.,).

Women with hyperprolactinemia, congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumours, diabetes mellitus Type 2, thyroid impairment, impaired renal or hepatic function, women on hormonal replacement therapy, medications' intake affecting insulin metabolism, and history of calcium or vitamin D supplementation were excluded from the study.

Women were divided into two groups: Group 1 consisted of 45 newly diagnosed women with PCOS while Group 2 consisted of 43 women without PCOS that acted as controls.

Ten millilitres of venous blood was drawn from each woman between 8:00-11:00 am after overnight fast during the follicular phase (second or third day of menstrual periods). Eight millilitres of blood samples were collected in plain tubes which were allowed to clot at room temperature for 30 minutes, then the samples were centrifuged at 2000 rpm for 10 minutes, the obtained serum was transferred to another tube (Six Eppendorf Safe-Lock tubes) and were frozen at –20°C to be analysed later, haemolyzed specimen were discarded. In addition, 2 ml of blood transferred to EDTA tubes were used for analysis of *VDR* gene polymorphism.

Each serum sample was analysed for measuring 25-hydroxy vitamin D, free testosterone, insulin, Luteinizing Hormone (LH), and Follicle Stimulating Hormone (FSH) by Enzyme-Linked Immunosorbent Assay (ELISA) using kit supplied by (Bioactive-Germany, Demeditec-Germany, Monobind-USA, Human Company-Germany) respectively, while calcium and glucose were measured by spectrophotometer using kits (Human Company-Germany). Additionally, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation [16];

HOMA-IR=Fasting plasma glucose (mg/dL)×Fasting plasma insulin (μU/mL)/405.

Besides, DNA samples were amplified and analysed for the Cdx2 (G/A) SNP of *VDR* gene.

The Cdx2 polymorphism (rs11568820) was genotyped through Allele Specific Multiple-Polymerase Chain Reaction (ASM-PCR) using predesigned specific primers as described in [Table/Fig-1]. Multiplex-PCR requires multiple primer sets within the same amplification reaction. This allows determining multi-DNA sequences within the same sample. In addition, the involvement of internal controls to evaluate the reliability of the PCR [17].

SNP	Location	Primers	Product size	Internal control
Cdx2 (G/A)	exone-1	**G-Forward:** 5`-AGG ATA GAG AAA ATA ATA GAA AAC ATT-3` **G-Reverse:** 5`-AAC CCA TAA TAA GAA ATA AGT TTT TAC-3`	G-110 bp	297 bp (G-forward A-reverse)
		A-Forward: 5`-TCC TGA GTA AAC TAG GTC ACA A-3` **A-Reverse:** 5`-ACG TTA AGT TCA GAA AGA TTA ATTC-3`	A-235 bp	

[Table/Fig-1]: Primer and product size for Cdx2 polymorphism of vitamin D receptor gene.

The reaction was performed in total volume of 50 μl using gold multiplex PCR preMix (Bioneer Corporation, Korea). The premix contained (DNA polymerase, pyrophosphatase and pyrophosphate, dNTPs, reaction buffer, tracking dye, and stabilizer. 25 μL of PCR premix, 6 μL of DNA sample, 1 μL of each forward and reverse primers and deionized sterile distilled water (DNase, RNase free) was added to complete reaction volume. PCR condition was performed under following conditions [Table/Fig-2].

No.	Steps	Temperature	Time	Cycles
1	Pre-denaturation	95°C	5 minutes	1
2	Denaturation	95°C	30 seconds	30
3	Annealing	60°C	30 seconds	30
4	Extension	72°C	1 minutes	30
5	Final extension	72°C	5 minutes	1

[Table/Fig-2]: PCR conditions.

The amplicons were electrophoresed with 100 bp ladder (Bioneer Corporation, Korea) to detect the size of fragments on 2% agarose gel. Gel visualised in Gel Documentation System/ UV transilluminator to reveal bands were interpreted as primers G-Forward and G-Reverse generate a 110 bp fragment when the *G*-allele is found while primers A-Forward and A-Reverse generate a 235 bp fragment when the *A*-allele is found. The primers G-Forward and A-Reverse generate a 297 bp fragment, which is considered as an internal control and is independent of the appearance of either the *G* or *A* allele [14].

STATISTICAL ANALYSIS

Data interpretation was carried out by using SPSS-24.0 (Statistical Packages for Social Sciences-version 24). Data was presented in simple measures of percentage, mean, and standard deviation. The significance of the difference of different means was assessed by using ANOVA test for difference among more than two independent means. Also, the significance of difference on different percentages was evaluated by using Pearson chi-square test. In addition, odds ratios with 95% confidence intervals were calculated to assess the strength of association between Cdx2 polymorphism of *VDR* gene and PCOS risk. Odds ratios were also calculated for allele frequency comparison (G vs A).

Hormonal and metabolic status in women with PCOS was assessed according to genotypic distributions of Cdx2 polymorphism. Moreover, vitamin D and calcium levels were calculated in respect with a genotypic distribution of Cdx2 polymorphism in both patients and controls.

RESULTS

There was no significant difference in genotypic frequencies distribution of Cdx2 polymorphism of *VDR* gene between women with PCOS and controls. In addition, odds ratio for GG carriers was 1.93 (95% CI=0.83-4.48), for AA carriers it was 0.95 (95% CI=0.31-2.93) while for GA carriers it was 0.52 (95% CI=0.22-1.23) as shown in [Table/Fig-3].

Genotype or Allele	PCOS (n=45)		Controls (n=43)		Odds Ratio	95% Confidence Interval	p-value
	No.	%	No.	%			
GG	24	53.3%	16	37.2%	1.93	0.83-4.48	0.141
GA	14	31.1%	20	46.5%	0.52	0.22-1.23	0.189
AA	7	15.6%	7	16.3%	0.95	0.31-2.93	NS
G-allele	62	68.9	52	60.5%	1.45	0.78-2.69	0.271
A-allele	28	31.1%	34	39.5%	0.69	0.37-1.28	0.271

[Table/Fig-3]: Allelic and Genotypic frequency distributions of Cdx2 polymorphism of vitamin D receptor gene among women with PCOS and controls.
*Significant difference between proportions using Pearson Chi-square test at 0.05 level; NS: Not significant

Additionally, metabolic and endocrine features of PCOS group were assessed by calculation of mean value of fasting serum glucose, insulin, HOMA-IR, LH, FSH, LH/FSH ratio, and free testosterone according to GG, GA, and AA carriers. The results were found significantly lower fasting serum glucose (p=0.02), insulin (p=0.01),

and HOMA-IR (p<0.001) in AA carriers than GA and GG carriers. Furthermore, levels of LH (p=0.002) and LH/FSH ratio (p=0.003) were significantly higher in GG carriers than GA and AA carriers (p=0.002), while no significant difference in the mean value of FSH (p=0.148) and free testosterone (p=0.091) was observed between GG, GA, and AA carriers as shown in [Table/Fig-4].

Parameters	GG (n=24)	GA (n=14)	AA (n=7)	p-value
	Mean±SD	Mean±SD	Mean±SD	
Fasting serum glucose (mg/dL)	95.02±2.85	93.32±4.08	89.97±1.67	0.02*
Fasting serum insulin (µU/mL)	14.25±1.23	14.4±1.06	12.4±0.604	0.01*
HOMA-IR	3.34±0.275	3.31±0.27	2.75±0.16	<0.001*
serum LH (IU/L)	10.17±2.27	7.48±2.35	7.51±2.23	0.002*
serum FSH (IU/L)	5.16±0.47	4.89±0.44	5.31±0.73	0.148
serum LH/FSH ratio	1.97±0.45	1.53±0.47	1.41±0.36	0.003*
Serum free testosterone (pg/mL)	5.27±0.47	5.50±0.51	5.04±0.12	0.091

[Table/Fig-4]: Mean value of fasting serum glucose, insulin, HOMA-IR, LH, FSH, LH/FSH ratio and free testosterone levels according to GG, GA, and AA carriers of Cdx2 polymorphism for patients group.
*Significant difference among more than two independent means using ANOVA test at 0.05 level.

Likewise, mean value of serum 25-hydroxy vitamin D and calcium levels were calculated according to GG, GA and AA carriers for both PCOS group and controls group. The results observed significantly lower levels of serum 25-hydroxy vitamin D in GG carriers than GA and AA carriers for both patients (p<0.001) and controls (p<0.001), with no significant difference in the mean value of calcium levels between GG, GA and AA carriers for patients (p=0.949) and controls (p=0.46) as demonstrated in [Table/Fig-5].

Parameters	A and/or G carriers	serum 25-OH vitamin D (ng/mL)	serum Calcium (mg/dL)
		Mean±SD	Mean±SD
PCOS	GG (n=24)	5.73±0.86	8.21±0.09
	GA (n=14)	8.1507±2.03	8.213±0.08
	AA (7)	10.74±2.76	8.2±0.081
p-value		<0.001*	0.949
Controls	GG (16)	13.71±3.33	8.93±0.253
	GA (20)	20.202±5.86	9.12±0.259
	AA (7)	24.22±5.46	9.15±0.19
p-value		<0.001*	0.46

[Table/Fig-5]: Mean value of serum 25-hydroxy vitamin D and calcium levels according to GG, GA, and AA carriers of Cdx2 polymorphism for patients and controls.
*Significant difference among more than two independent means using ANOVA test at 0.05 level.

DISCUSSION

In the present study, Cdx2 (G/A) single nucleotide polymorphism of the *VDR* gene was examined among women with PCOS and assessed for their effect on vitamin D levels and/or PCOS manifestations. The results found no significant difference in genotypic frequency distributions between women with PCOS and controls and suggested non significant association between Cdx2 polymorphism and PCOS risk. In this regard, an Austrian study showed no significant difference in the genotype frequencies of Cdx2 polymorphism between PCOS patients and controls but found an association between Cdx2 polymorphism and insulin metabolism [18]. In contrast, an Indian study found significant difference in the genotype and allele frequency distributions of the Cdx2 polymorphism between patients with PCOS and controls and suggested protective effect of Cdx2 polymorphism against PCOS manifestations [19].

A study by Zadeh-Vakili A et al., suggested that genetic variation of the VDR was associated with severity of clinical manifestations of PCOS; however, not with risk of the disease [20]. While a recent study by Siddamalla S et al., found an association between *VDR* gene polymorphisms and PCOS risk [21].

Prior evidence also proposed that *VDR* gene polymorphisms were found to be associated with vitamin D deficiency as well as metabolic and endocrine abnormalities seen in PCOS [18,22]. In addition, the pathogenesis of PCOS has been associated with impacts of (TaqI, BsmI, FokI, ApaI andCdx2 polymorphisms) of *VDRs* gene on LH and SHBG levels [23], testosterone levels [18], serum insulin levels and IR in women with PCOS [3,18,22].

The concept that active vitamin D and its receptor were included in metabolic and endocrine disturbance seen in PCOS is assisted by the fact that the VDRs manage more than 3% of the human genome, involving genes that are critical for glucose homeostasis [24]. More precisely, VDR is found in 2776 genomic positions that alter the expression of 229 genes in various tissue, including skeletal muscle, brain, breast, pancreas, parathyroid glands, immune cells, cardiac cell, ovaries [24-26], pituitary gland, and human endometrium [27]. Thus, vitamin D affects a wide range of physiological processes [24].

Besides the expression of the VDR in beta cells of pancreas might illustrate impact of vitamin D on insulin secretion [28]. Also, the presence of Vitamin D Response Element (VDRE) in the insulin receptor gene explains the mechanism by which vitamin D deficiency could influence insulin sensitivity [29]. This evidence was supported by prior research which found significant associations between *VDR* gene variants and insulin sensitivity [30].

The findings of the present study showed significantly lower serum 25-hydroxy vitamin D levels and higher LH and LH/FSH ratio for individual with GG genotype than others with AA and GA genotype. Also, results showed odds ratio for *G*-allele of Cdx2 polymorphism was 1.45 (95% CI=0.78-2.69) and for GG carriers 1.93 (95% CI=0.83-4.48), which indicate nearly two fold increased risk of disease for GG carriers than AA and GA carriers. These results agree with previous studies [12,31] which identified that *G*-allele of Cdx2 polymorphism is accountable for decreasing of transcriptional activity of *VDR* gene. Thus, lower transcriptional activity of VDR alters several functions managed by active vitamin D [31].

Additionally, the result of the present study revealed that lower levels of fasting serum glucose, insulin, and HOMA-IR for PCOS cases with Cdx2-AA genotype than GG or GA genotype which is similar to another study by Wehr E et al., [18]. This may be clarified by previous studies which supposed that Cdx2-*A* allele carriers have higher intestinal calcium absorption due to increased expression of intestinal calcium channel protein [32]. Calcium has important role in the regulation of insulin secretion by pancreatic beta cells. Thus, *VDR* gene polymorphism may affect beta cell of the pancreas [18].

Recent study by Reis GV et al., suggested that Cdx2 polymorphism is the variant with probable link with the susceptibility as well as severity of this syndrome, involving greater IR, fasting insulin, testosterone levels, body mass index, lower vitamin D levels, and exhibits more significant odds ratio values when PCOS and control groups are compared. Moreover, serum vitamin D levels have a greater effect on PCOS manifestations compared to *VDR* gene polymorphisms, as vitamin D deficiency has an impact on severity of the PCOS and influences metabolic as well as anthropometric parameters. Furthermore, vitamin D supplementation had an influential role in the management of PCOS features, enhancing the contribution of vitamin D deficiency in the pathogenesis of this syndrome [33].

LIMITATION

In terms of limitations, a small sample size together with the single *VDR* gene polymorphism was examined. These limitations may be improved by increasing sample size as well as studying additional SNPs of *VDR* gene such as TaqI, BsmI, FokI, and ApaI polymorphisms.

CONCLUSION

Cdx2 polymorphism of vitamin D receptor gene has an association with severity of clinical features in PCOS, however not with risk of development of the disease meaning that genetic variation is not directly linked to risk of this syndrome but may indirectly affect disease development via regulation of vitamin D levels.

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