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Assessment of *Cdx2* polymorphism in Iranian women with polycystic ovary syndrome

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Abstract

Background Women's fertility is affected by polycystic ovarian syndrome (PCOS) as an endocrine disorder with characteristic symptoms such as insulin resistance, polycystic ovaries, menstrual irregularities, and obesity. In polycystic ovarian syndrome, the vitamin D endocrine system is regulated by the vitamin D receptor (VDR) associated with type II diabetes, endocrine dysfunctions, and insulin resistance. Therefore, the current paper deals with the investigation of the connection between *Cdx2* VDR gene polymorphism and the biochemical factors in obese PCOS women.

Material and methods In the current case–control study, 40 obese women without PCOS and 38 obese women with PCOS were enrolled in May–September 2016. Insulin, IGF1, FBS, and HOMA-IR were examined for the participants along with the allelic and genotypic frequency of *Cdx2* polymorphism G/A (rs11568820) from Isfahan Fertility and Infertility Center, Iran. The ASM-PCR (multiplex allele-specific PCR) technique was utilized in this regard.

Results The age of PCOS women was less ($P < 0.001$) than the controls. In PCOS women, insulin, FBS, and HOMA-IR serum levels were higher than in the control women (all *P* values 0.05). For GG, AG, AA,A, and G *Cdx2*(A/G) genotypic/allelic frequencies were 84.2%, 15.8%, 0%, 7.9%, and 92.1% in cases and 87.5%, 12.5%, 0%, 6.3%, and 93.8% in controls, respectively. HOMA-IR ($P = 0.047$ and $P = 0.033$, respectively) and insulin than those with the AG genotype were in PCOS women with the GG *Cdx2* genotype. The highest IGF-1 mean value ($P = 0.020$) was found for the AG genotype in PCOS. In our study, a significant relation was found only between PCOS and FBS, in terms of a logistic regression analysis of *Cdx2* and parameters.

Conclusion In the present study, it was indicated that the GG genotype in PCOS subjects was associated with the IGF-1, HOMA-IR, and insulin. Similarly, no association was found between obese PCOS patients and *Cdx*2 in the 1a promoter area of the VDR gene in our study.

Keywords Polycystic ovarian syndrome, Obesity, Gene polymorphism, Vitamin D receptor

Introduction

Polycystic ovarian syndrome (PCOS) is the most common heterogeneous endocrine, reproductive, and metabolic disorder in reproductive-age females. It influences more than 20% of premenopausal women, thus causing this syndrome potentially [1–3]. Infertility and anovulation are more prevalent in PCOS women [4]. About half of all infertile women are affected by obesity. Menstrual irregularities and obesity seem to be correlated directly. This is caused by adipose tissues as the greatest peripheral area for the androgen-to-estrogen aromatization contributing to the formation of estrogen

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[5]. PCOS women possess higher anxiety, depression, and perceived stress levels compared to women without those conditions. Stress can affect the relationship between depression, PCOS, and anxiety [6]. In adolescence, PCOS should be treated and diagnosed at the initial stages when there is a menstrual disorder as it can lead to reproductive, oncological, and metabolic complications.

Diet, drugs, and lifestyle variations are some of the treatment options [7]. By accumulating ovarian follicles in different stages of atresia or maturation, the ovaries become polycystic, which is common in PCOS patients. PCOS includes multigene and complex etiology affected by lifestyle and epigenetic factors. However, its main cause is not known [2]. Anovulatory infertility is almost caused by PCOS related to hyperinsulinemia, dyslipidemia, insulin resistance (IR), and central obesity [8, 9]. These factors are all connected to type II diabetes, cardiovascular diseases, and metabolic syndrome. Metabolic disturbances are common in women with PCOS [8]. It was currently evidence that hyperandrogenism contributes significantly to PCOS pathogenesis, resulting in metabolic and reproductive problems [10]. We considered insulin-like growth factor 1 (IGF-1), i.e., a polypeptide hormone with a structure the same as insulin. Vitamin D as a fat-soluble vitamin is vital to bone health. Interacting with the genome, it produces nonracemic and calcemic effects after conversion into several biologically active metabolites in the body. It was highly revealed that cellular proliferation, immune function, differentiation, insulin secretion, vascular tone, and fertility are affected by vitamin D [11–13] vitamin D (a secosteroid hormone), its receptor, and metabolizing enzymes are included in vitamin D endocrine system, which contributes to the biologically active form of hormone [14]. The vitamin D receptor (VDR) regulates over 3% of the human genome including glucose metabolism genes, which supports this theory [15]. Though, limited information is available on the role of vitamin D metabolism gene variants in PCOS.

Vitamin D and VDR variants, such as *Apa*I, *Fok* I, *Cdx2*, and *Taq*I, are connected to endocrine, genetic, and metabolic aspects of PCOS. Thus, their important role is indicated [16, 17]. In PCOS women, these variants are connected to insulin sensitivity and secretion [17, 18]. Yamamoto et al. identified a functional binding site for the intestine-specific transcription factor *Cdx2* in the VDR gene 1a promoter region [19]. VDR polymorphisms are related to metabolic and endocrine features of PCOS, including hyperandrogenism and insulin resistance [17, 20–22]. Thus, the present work aimed to assess the possible relations among the *Cdx2* variant of the VDR gene, IGF-1, insulin, HOMA-IR, and FBS of obese PCOS women among Iranian samples.

Materials and methods

Study population

In May–September 2016, a case-control study was performed, on 38 obese PCOS women and 40 obese women without PCOS in Isfahan Fertility and Infertility Center (IFIC), Isfahan, Iran. The two groups had a mean age of 18–40 years old and a body mass index (BMI) ≥ 30. The menstrual disorders included oligomenorrhea (6 or fewer menses per year), amenorrhea (no menses over the past 6 months), and hyperandrogenism (biochemical and clinical signs), like alopecia, hirsutism (Ferriman-Gallwey score ≥ 6), or acne, along with the increased androgen levels (normal range testosterone < 0.77 ng/ml and normal free testosterone level < 3.18 pg/ml). Obese PCOS women with married/infertile, BMI ≥ 30, without a family history of PCOS, were the subjects of the study group. However, obese women with BMI ≥ 30 were included in the control group. They were married (with or without children) without PCOS family history. PCOS women and the control group on medications were excluded, influencing the calcium metabolism, carbohydrate, endocrine system, or lipid for over 3 months prior to the study. Height, BMI, and weight were recorded, and the exact measurements of weight (kg) and height (meters) were performed to calculate the BMI under a nutritionist's supervision.

Biochemical measurements

The blood samples were gathered for hormonal assays in the follicular phase followed by an overnight fast, for 2 to 6 days of a menstrual cycle or over a spontaneous bleeding episode in controls along with a menstrual cycle induced by progestin in PCOS patients. Fasting blood sugar (FBS) levels were determined in the serum using a spectrophotometer (#95014 Roche Hitachi 912 chemistry analyzer, Pars Azmoon, IRAN). To determine insulin levels, a chemiluminescence immune assay (CLIA) kit (#017039XB Immune analyzer Liazon, Diasorin, Germany) was utilized. The homeostasis model assessment of insulin resistance (HOMA-IR) was measured using the product of fasting insulin and glucose levels divided by 22.5. To detect IGF-1, the enzyme immunoassay technique (#301115 State fa) was utilized. Using the enzyme immunoassay method (#301115 State fax 2100, Mediagnost, Germany), IGF-1 was calculated.

Genotype analysis

The gathered blood samples were kept at 4°C in tubes comprising EDTA for DNA analysis. To purify genomic DNA from the whole blood, an isolation kit was used (Iraizol#1004, RNA Biotechnology Company, Iran). Using ASM-PCR (multiplex allele-specific PCR), we considered the *Cdx2* polymorphism G/A(rs11568820). The

ASM-PCR test was performed using two primer sets, as follows:

(G For:5′ AGGATAGAGAAAATAATAGAAAAC ATT3′; G Rev:5′ AACCCATAATAAGAAATAAGTTTT TAC3′; A For:5′ TCCTGAGTAAACTAGGTCACAA3′; A Rev:5′ACGTTAAGTTCAGAAAGATTAATTC3′).

A BIO-RAD system was used to perform the PCR amplification (T100TM Thermal Cycler). The PCR reaction system's total volume was 25 μ*L*, with 12.5 μ*L* of master mix 1X (# A180306, AMPLIQON, Denmark). This work was performed using 9.5 μ*L* of genomic DNA, 0.5 μ*L* of *Cdx2* primers (A-For, A-Rev, G-For, G-Rev), and 1 μ*L* of *Cdx2* primers (A-For, A-Rev, G-For, G-Rev). Then, after denaturation at 95°C for 5 min, 28 denaturation cycles happened at 95°C for 30 s, annealed at 59°C for 45 s until 72°C in PCR for 30 s. The final extension lasted for 5 min at 72°C.

Using 2.5% agarose gels, PCR products were resolved. Three PCR fragments were produced by these four primers including (1) G-For and G-Rev primer set, amplifying the G allele (110 bp); (2) A-Rev and A-For, amplifying the A-allele (235 bp); and (3) out-primer pair (A-Rev and G-For), amplifying the internal control PCR fragment (297 bp).

Statistical analysis

To compare the distribution of the genotype and allele frequencies and determination of Hardy-Weinberg equilibrium for *Cdx2* polymorphism, chi-square test (χ^2) was used. Using SPSS Inc. was used (Chicago, Illinois, USA, SPSS V.20.0), and χ^2 value was calculated. Moreover, to determine the differences in control and case characteristics (P<0.05), Mann-Whitney test and independent t test were performed considering the statistically significant results.

Results

The clinical and biochemical features of the participants are shown in Table 1. The PCOS group's age was less than the controls (P<0.001). FBS, insulin, and HOMA-IR levels in the blood were all higher than the controls (all Ps<0.05) in PCOS patients. No considerable differences were found in clinical properties such as BMI and IGF1 (P>0.05) between PCOS women and controls.

Table 2 shows the allelic and genotypic frequencies of *Cdx2* VDR polymorphism in Exon 1e in obese PCOS women and control women. There was Hardy-Weinberg equilibrium in the VDR *Cdx2* genotypic distribution in control women ($\chi^2 = 0.177$, $P = 0.673$).

The A and G alleles' frequencies of *Cdx2* polymorphism were 6.3% and 93.8% in controls and 7.9% and 92.1% in cases. Likewise, the frequencies of AA, GG, and AG genotypes of the *Cdx2* variant were 0%, 87.5%, and 12.5%, in controls and 0%, 84.2%, and 15.8%, in cases, respectively. There was no significant difference between obese control women and obese PCOS patients, genotypes ($P = 0.677$), and allele ($P = 0.688$), frequencies.

Table 1 The clinical characteristics of control and PCOS women

	PCOS	Control	*P* value
Number	38	40	-
Age (year)	28.58 ± 5.83	34.37 ± 5.07	P< 0.001[a]
BMI (kg/m^2)	32.39 ± 3.47	33.82 ± 3.37	0.072[a]
FBS	96.21 ± 10.20	89.47 ± 6.41	0.001[a]
Insulin	24.57 ± 14.37	18.02 ± 10.36	0.021[b]
IGF-1	6.70 ± 5.55	6.02 ± 2.52	0.582[b]
HOMA-IR	5.97 ± 3.73	4.04 ± 2.48	0.01[b]

All differences were considered significant at $p < 0.05$

BMI body mass index, *FBS* fasting blood sugar, *IGF-1* insulin-like growth factor-1, *HOMA-IR* homeostatic model assessment of insulin resistance

[a] Independent sample *t* test and [b]Mann-Whitney test

Table 3 shows the biochemical traits based on *Cdx2* polymorphism genotypes. Among the *Cdx*2 genotypes (AG and GG) in the patient group, out of the 5 biochemical traits examined HOMA-IR, IGF-1 mean, and insulin levels were significantly different. PCOS women with the GG *Cdx2* genotype had a higher mean of insulin and HOMA-IR (P=0.047 and P=0.033, respectively) compared to the AG genotype. The highest mean IGF-1 level (P=0.020) was for the AG genotype in PCOS patients. In the control group, there were no considerable differences in the biochemical parameters between the two genotypes. Furthermore, there were no significant differences in the mean of FBS in the two groups of GG and AG in the PCOS women group.

Table 4 displays the association between FBS, IGF-1, insulin, and the *Cdx2* polymorphism with PCOS utilizing logistic regression analysis. We found that only FBS and polycystic ovary syndrome were significantly different. Thus, by increasing the blood sugar, the PCOS probability significantly increases.

Discussion

In the present study, *Cdx*2 polymorphism was studied among obese women with PCOS and without it. The association of *Cdx2* variant with IGF-1, FBS, insulin, and HOMA-IR phenotype was also assessed. FBS, insulin, and HOMA-IR serum levels were all higher in PCOS women, compared to the controls (all Ps < 0.05). We found the common GG genotype as the most common in PCOS subjects and control. The lowest frequency was for the AG genotype in both groups. In no groups, no AA individual was found. There were no significant differences between the patient and control groups in A and

Table 2 Genotypic and allelic frequencies of *Cdx2* polymorphism; Hardy–Weinberg equilibrium in the control group

	PCOS number (%)	Control number (%)	*P* value	χ^2
Genotype				
GG	32 (84.2)	35 (87.5)	0.677	
AG	6 (15.8)	5 (12.5)		
AA	0	0		
Total	38 (100)	40 (100)		
Allele				
A	6 (7.9)	5 (6.3)	0.688	
G	70 (92.1)	75 (93.8)		
Total	76 (100)	80 (100)		
Hardy–Weinberg equilibrium in the control group	-	-	0.673	0.177

All differences were considered significant at $p < 0.05$. χ^2 chi-square test

Table 3 Biochemical traits according to genotypes of *Cdx2* polymorphism

Variable	Group	*Cdx2* genotype				*P* value[a]
		Number	GG	Number	AG	
FBS	PCOS	32	97.19 ± 10.60	6	91.00 ±5.90	0.155
	Control	35	89.80 ±6.61	5	87.20 ±4.66	0.448
Insulin	PCOS	31	26.14 ±14.74	5	14.84 ±6.27	**0.047**
	Control	35	18.33 ±10.65	5	15.82 ±8.69	0.668
IGF-1	PCOS	32	5.66 ±4.33	6	12.25 ±8.25	**0.020**
	Control	35	5.93 ±2.61	4	6.79 ±1.46	0.487
HOMA-IR	PCOS	31	6.40 ±3.82	5	3.30 ±1.50	**0.033**
	Control	35	4.13 ±2.57	5	3.41 ±1.85	0.759

Data are the mean ± SD. All differences were considered significant at $p < 0.05$

FBS fasting blood sugar, *IGF-1* insulin-like growth factor-1, *HOMA-IR* homeostatic model assessment of insulin resistance

[a] *P* value based on Mann–Whitney test

Table 4 Logistic regression analysis between FBS, insulin, IGF-1, and *Cdx2* with PCOS

Biochemical parameters and *Cdx2*	Odds ratio	Lower 95%CI	Upper 95%CI	*P* value
FBS	1.090	1.016	1.170	**0.016**
Insulin	1.024	0.979	1.070	0.300
IGF-1	1.033	0.914	1.167	0.604
Cdx2	2.059	0.446	9.504	0.355

Data presented as an odds ratio and 95% CI (95% confidence interval). $P < 0.05$ was considered statistically significant. Logistic regression

FBS fasting blood sugar, *IGF-1* insulin-like growth factor-1

G genotypes or alleles. For other populations, various results were found. Meyer and Bornman (2018) revealed that the AA genotype ($P < 0.001$) was more dominant in Blacks than Whites. Additionally, lower induction of VDR mRNA (P<0.050) was represented by AA vs. AG/GG along with lower 25(OH)D3 levels (P<0.010) and higher promoter methylation of VDR (P<0.050), in primary monocyte/macrophages [23]. Fang et al. represented the frequency of the AA genotype as zero. The AA genotype frequency is zero among white populations of the Middle East (Table 2) [24]. For the *VDR* gene *Cdx*2 (G/A), *Cdx2* is a transcription factor, resulting in a *VDR* gene with a defective binding area for the CDX2 transcription factor, which is found only in the intestine [19]. In Korea, a study was conducted on 432 PCOS women and 927 control women without PCOS. No significant relation was found in this work, between the increased insulin levels and insulin sensitivity as well as the *Cdx2* polymorphism in PCOS women followed by multiple linear regression [25].

Rana Ali Hamdi et al. [26] indicated a relation between the *Cdx2* polymorphism of the VDR gene *and* the clinical characteristics severity in PCOS without the risk of a disease development. It was revealed that the risk of

developing a syndrome and genetic variation are not connected directly. However, it may have direct effects on the disease development by vitamin D levels regulation.

This study was conducted on 45 PCOS and 43 non-PCOS women in Baghdad. In reproductive results, *VDR* SNPs could play a key role in coding regions of ovarian signaling pathways related to synthesize hormone receptors, transport molecules, and metabolic enzymes. A statistically significant association was found between VDR polymorphisms and clinical/biochemical PCOS determinants in some studies. According to Dasgupta et al., *Fok*I polymorphism was related to infertility. However, there was relation between the *Cdx*2 polymorphism and testosterone levels in PCOS [27]. The relation between polymorphisms (*Apa*I, *Fok*I, *Cdx2*, and *Bsm*I) was considered by Szafarowska et al. within AMHR2 (type-II anti-Müllerian hormone receptor), the VDR gene, and the AMH (anti-Müllerian hormone) genes and their effects on AMH and 25(OH)D levels in 75 PCOS patients and 23 healthy women. There was a dramatically higher homozygous GG genotype frequency, based on *Fok*I (rs2228570), VDR *Cdx2* polymorphism, and *Apa*1 (rs7975232), in the PCOS group than in the control group ($P<0.05$). The higher AMH levels in PCOS women were responsible for the VDR polymorphisms [28].

A study was performed on the relationship between *Taq*I and *Bsm*I polymorphisms among PCOS-obese subjects in a population in Iran. It was indicated that *Taq*I and BsmI polymorphisms were not associated with obesity accompanied by PCOS susceptibility [29, 30]. No statistically significant relation was found between the PCOS risk and polymorphisms within vitamin D pathway-related genes in a large case-control study in Pakistan, though numerous studies have been conducted on the *Cdx2* polymorphism variant [31]. There were no significant differences in genotypic and allelic frequency distributions of *Cdx2* between PCOS cases and controls in our study, which is consistent with the previous studies in Pakistan and Austria [32, 33].

Substantially higher TaaI/*Cdx*2 GG genotype frequency was revealed by Lesiak et al. [34] among psoriasis patients in 2021, related to IL-17 and IL-23 concentration. Furthermore, TaaI/*Cdx*2 AA may have a role in the psoriasis patients' phototherapy response. Higher levels of insulin and HOMA-IR were found in the present study in GG genotype subjects than the GA in both obese women and PCOS control. This is consistent with the results of Wehr et al. [33]. However, our results were significant only in the PCOS group. However, this polymorphism has no clear role in metabolic parameters. Two different hypotheses can be used to explain the relationship between these metabolic parameters and variants. First, in β cells, the calcium flow is well-defined for insulin secretion. Second, the VDR gene is a candidate gene for β cell function since vitamin D as the primary hormone has a role in calcium regulation. Moreover, vitamin D increases the glucose transport response by stimulating the insulin receptor gene expression. Vitamin D response elements (VDRE) are found in the human insulin gene promoter and 1,25(OH)D2 is responsible for the transcription of the insulin gene [35].

No exact mechanism has been found for PCOS owing to its multifactorial nature (genetic and environmental). Genetic variants are believed to be effective in different signaling pathways in the women's reproductive process. The difference in the results of research about PCOS can be caused by the different lifestyles (exposure to sunlight, consumption of different foods, physical activity, etc.) accompanied by the genetic variants of various genes in different countries. PCOS can be predicted more definitely worldwide with genetic variants by determining this relationship more reliably and accurately in different countries.

The small sample size and a single VDR gene polymorphism were among the limitations of the present study, which can be overcome by further studies on SNPs of the VDR gene and other genes included in polycystic ovary syndrome, such as *FTO*.

Conclusion

No association was concluded between obese PCOS patients and *Cdx2* (rs11568820) in the VDR gene 1a promoter region in the present work, though a specific genotype (GG) in PCOS subjects was associated with the biochemical parameters such as insulin, IGF-1, and HOMA-IR.

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Authors' contributions

MO and HN conceived and designed the study and collected, analyzed, and interpreted the data. BK performed the laboratory protocols of the research. BK, MO, and HN contributed to the drafting. MO and HN performed critical revision of the manuscript. BK and MO contributed to revising the work. All authors have approved the final version submitted for publication and take responsibility for the statements made in the published article. MO is the corresponding author.

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As the study is for student research, it has a personal financial source. This article extracted from M.Sc. Thesis (Behdis Khansari).

Availability of data and materials

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

In our study, all steps performed involving humans were in accordance with the 1964 Helsinki Declaration. The Research Committee of the Falavarjan

Branch, Islamic Azad University, Isfahan, Iran, approved this study (Approval code: 17230520942008).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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