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Original Article

Vitamin D receptor 1a promotor −1521 G/C and −1012 A/G polymorphisms in polycystic ovary syndrome

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Abstract

Objective: The aim of this case-control study was to investigate whether the vitamin D receptor (*VDR*) *1a* promoter gene polymorphisms are associated with susceptibility to polycystic ovary syndrome (PCOS).
Methods: Women with PCOS and a control group, all aged 18–45 years, were enrolled. Genotypes of two functional single nucleotide polymorphisms (SNPs), the 1521 bp (G/C) and 1012 bp (A/G), located on the 1a promoter of the *VDR* gene were determined by using direct sequencing. Serum 25-hydroxyvitamin D levels were measured by ELISA.
Results: Two functional SNPs in the 1a promoter region of the *VDR* gene were in complete linkage disequilibrium. The genotype distributions of these two polymorphisms in the PCOS group were not significantly different from those of the control group. Further subgroup analyses according to body mass index also revealed no significant differences in the genotype distribution in the PCOS group. Significantly lower serum 25-hydroxyvitamin D levels were observed in the heterozygous 1521CG/1012GA haplotype of both groups. Metformin treatment was only effective to increase serum 25-hydroxyvitamin D levels in PCOS patients carrying the homozygous 1521G/1012A haplotype.
Conclusion: These results suggest that the *VDR 1a* promoter polymorphisms may not be associated with the risk for PCOS, but are associated with serum 25-hydroxyvitamin D levels. Metformin treatment will be beneficial to PCOS patients without the *VDR 1a* promoter variant in Taiwanese population.

Keywords: 25-hydroxyvitamin D; metformin; polycystic ovary syndrome; single nucleotide polymorphism; vitamin D receptor

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disease in reproductive women with a complex hormonal disturbance that affects the menstrual cycle and leads to metabolic consequences in later life [1]. PCOS is associated with obesity, insulin resistance, and increased risk of type 2 diabetes mellitus, dyslipidemia, cardiovascular disease, and endometrial carcinoma. It is known that vitamin D is involved in the pathogenesis of type 2 diabetes because it affects insulin metabolism [2]. Vitamin D needs to be converted to its active form and activates vitamin D receptor (VDR) to regulate target gene expression. Serum concentration of 25-hydroxyvitamin D is considered to be the most reliable indicator of clinical vitamin D status, and a circulating 25-hydroxyvitamin D level <80 nM (32 μg/L) is defined as vitamin D deficiency [3]. Vitamin D is also thought to play a role in the pathogenesis of

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PCOS. Serum 25-hydroxyvitamin D levels are negatively correlated with body mass index (BMI), indices of insulin resistance, and leptin levels in PCOS women [4]. In addition, PCOS women with features of metabolic syndrome have lower serum 25-hydroxyvitamin D levels than PCOS women without these features [5]. However, the relationship between PCOS and vitamin D deficiency is inconsistent from these studies. Although low 25-hydroxyvitamin D levels are associated with increased risk for various disorders, such as cardiovascular disease, diabetes, and metabolic syndrome, the therapeutic benefits of vitamin D supplementation require further exploration [6]. In obese PCOS women, vitamin D analogue treatment was found to increase the first phase of insulin secretion significantly and to improve the lipid profile [7].

PCOS is characterized by anovulation, hyperandrogenism, and polycystic ovarian morphology. Ultrasound examination, especially 3D ultrasonography, may help in the evaluation of ovarian morphology and the diagnosis of PCOS [8]. Menstrual abnormality is also an important characteristic in the diagnosis of PCOS. Laparoscopic ovarian drilling or gonadotropin stimulation is recommended as a second-line therapy for clomiphene citrate-resistant anovulatory PCOS women under metformin treatment. Our previous study found that this surgical procedure may help to improve endocrine profiles and intraovarian stromal flow in patient with PCOS [9]. However, it has the risk of decreased ovarian reserve and adhesion formation. Some PCOS patients can normalize their menstrual cycles after calcium plus vitamin D supplementation [10]. Vitamin D replacement treatment may also improve metabolic parameters and menstrual frequency in PCOS women [11]. The combination of calcium–vitamin D and metformin in the infertility treatment of PCOS patients demonstrated a better response in dominant follicular development and possible improvement of menstrual irregularities than either drug alone [12]. However, as these findings were based on a small scale of study (small sample size), further studies using larger sample sizes should be warranted.

Although familial aggregation indicates a genetic susceptibility to PCOS, the genetic etiology of PCOS remains unknown. There are large interindividual differences in the signs and symptoms among women with PCOS. Interaction of multiple genetic factors with environmental, particularly nutritional factors, might contribute to the heterogeneity of clinical and biochemical features in PCOS [13]. There are also ethnic and racial variations in the prevalence of PCOS and its symptoms. Some subtle DNA sequence variations (single nucleotide polymorphisms, SNPs) may occur more frequently in the population of PCOS, which may change the formation of protein complexes and the biologic effects. Our previous result showed different inflammatory responses among PCOS patients with insulin receptor substrate-2 polymorphisms in the Taiwanese population [14]. Interleukin-6 may be considered as an early low-grade chronic inflammatory marker among PCOS patients with the insulin receptor substrate-2 *Gly1057Asp* polymorphism. IL-6 was significantly elevated in PCOS women, especially in the insulin receptor substrate-2 homozygous *Asp* population, and significantly decreased after metformin treatment in this homozygous *Asp* variant.

Mahmoudi suggests that the *VDR* polymorphisms may be associated with phenotype development and insulin resistance in PCOS women [15]. In an evolutionary comparison study, *VDR 1a* promoter is highly conserved among mammals. There are two functional SNPs in the 1a promoter region of the *VDR* gene [16], the 1521 bp (G/C) and 1012 bp (A/G), located within a GATA site with human specificity [17]. In this study, we hypothesized that these two functional SNPs may influence VDR expression and might then be associated with susceptibility to the phenotypes of PCOS. We used candidate gene polymorphism investigation to find the correlation and effects of *VDR 1a* promoter SNPs in PCOS patients in a Taiwanese population. This study may facilitate the establishment of a domestic gene polymorphism databank via SNP study to provide more detailed genetic information for PCOS, and to understand the functional consequences of these variations.

Materials and methods

Patients

From November 1, 2007 to January 31, 2009, a hospital-based study was conducted to investigate the association between *VDR 1a* promoter SNPs and PCOS. A study group of 188 women with PCOS were enrolled, along with 143 women without any evidence of PCOS as a control group; the women of both groups were aged 18–45 years. The patients were observed for routine gynecological examination at National Cheng Kung University Hospital, Tainan, Taiwan. The study was approved by the hospital's institutional ethics committee and informed consent was obtained from each patient.

Patients with PCOS were diagnosed according to the 2003 Rotterdam ESHRE/ASRM criteria [18]. The diagnosis was based on the presence of at least two of the three following criteria: oligomenorrhea and/or amenorrhea; clinical and/or biochemical evidence of hyperandrogenism; and polycystic ovaries determined by ultrasonography with exclusion of other etiologies. The control group of women without PCOS had regular menstrual cycles, and no evidence of hyperandrogenism or polycystic ovaries.

Clinical, biochemical, and ultrasonographic evaluation was performed in each participant. Biochemical laboratory parameters at baseline and 3-month metformin administration were evaluated after an overnight fast and a 75-g 2-hour oral glucose tolerance test. Biochemical assessment consisted of complete hormonal, including serum follicle stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, prolactin, estradiol, 17-OH-progesterone, total testosterone, and sex-hormone binding globulin; metabolic evaluation included evaluation of lipid, glucose, and insulin levels. Insulin resistance was evaluated using the homeostasis model analysis [fasting glucose (mg/dL) × fasting insulin (μU/mL)/405], the quantitative insulin-sensitivity check index {1/[log(fasting insulin) + log(fasting glucose)]}, and the fasting glucose-to-insulin ratio (A/I). When women with PCOS had

insulin resistance, metformin was administered as 500 mg tablets with the same regimen (3 tablets daily) for 12 weeks.

Genotyping

A 10 mL whole blood sample was taken from each participant for genotyping. Genomic DNA was extracted from whole blood using the GeneMark extraction Kit (GeneMark Technology Co., Ltd., Tainan, Taiwan) according to the manufacturer's instructions. Genotyping of the two promoter SNPs [rs7139166 (−1521 C>G), rs4516035 (-1012 T>C)] of the human *VDR* gene were performed by using direct sequencing. PCR reaction was carried out using primers forward 5′-TGC AGA GAA TGT CCC AAG GT-3′ and reverse 5′-GTC CTG CCA GTC TGA TGG AT-3′ for the −1521 C>G polymorphism, and primers forward 5′-AGC AGA TTT GCT GGG CTC TA-3′ and reverse 5′-TGC TTC CCT TGA CTG TGT GA-3′ for the −1012 T>C polymorphism. The PCR reactions were performed in 96-well microtiter plates and the sequencing reactions were performed using the ABI BigDye Terminator reagents (Applied Biosystems, Foster City, CA, USA). The PCR products were sent to the Nucleic Acid Sequencing Center of National Cheng Kung University for sequencing using the ABI 3100 DNA sequencer (Applied Biosystems). The sequence data were analyzed by using the PolyPhred software (v5.04) [19]. The genotypes were assigned to the participants independently by two individuals blinded to the participant information.

Enzyme immunoassay for the quantitative determination of serum 25-hydroxyvitamin D levels

The commercial 25-hydroxy Vitamin D enzyme immunoassay kit (Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA) was used for quantification of the serum levels of 25-hydroxyvitamin D according to the manufacture's protocol. The diluted samples with biotin-labeled 25-hydroxyvitamin D were incubated with a highly specific sheep 25-hydroxyvitamin D antibody for 2 hours at room temperature. Horseradish peroxidase-labeled avidin was added to bind selectively to complexed biotin. After color was developed using a chromogenic substrate, the absorbance of each well at 450 nm (reference 650 nm) was read in a plate reader. A standard calibration curve of signals was used to calculate the 25-hydroxyvitamin D concentration of unknown patient samples. The sensitivity of 25-hydroxyvitamin D assay was 5 nM, and intra- and interassay coefficients of variance were 5.3% and 4.6%, respectively.

Statistical analysis

Continuous results are expressed as mean ± SEM. A two-sample *t* test was used to evaluate the clinical variables between patients and controls. One-way analysis of variance was used to evaluate the parameters among three groups. A paired *t* test was used to evaluate the 25-hydroxyvitamin D changes before and after metformin therapy in PCOS patients. Pearson's χ^2 tests were used to test for the difference of allelic and genotypic frequencies of *VDR 1a* promoter SNPs between groups. Logistic regression analysis was performed to evaluate an association between specific *VDR 1a* promoter alleles and their susceptibility to PCOS. Odds ratios (ORs) and 95% confidence interval (95% CI) from the logistic regression model were used to estimate the magnitude of the association between the *VDR 1a* promoter genotype and PCOS. Statistical analyses were performed using the SPSS program (Version 15.0, SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

Clinical and biochemical characteristics of the study population

The PCOS patients had significantly higher body weight (61.62 ± 1.06 kg) and BMI (24.17 ± 0.39 kg/m^2) than the control women (56.48 ± 0.87 kg for body weight; 22.06 ± 0.32 kg/m^2 for BMI). Additionally, the levels of luteinizing hormone and testosterone were significantly higher in the PCOS group than in the control group. The PCOS patients had significantly elevated levels of fasting insulin, 2 hour glucose, and 2 hour insulin during the OGTT (fasting insulin: 8.94 ± 0.63 μU/mL; 2 hour glucose: 112.06 ± 4.65 mg/dL; 2 hour insulin: 61.87 ± 4.65 μU/mL) compared with the control participants (fasting insulin: 6.41 ± 1.15 μU/mL; 2 hour glucose: 94.75 ± 2.73 mg/dL; 2 hour insulin: 26.69 ± 1.90 μU/mL; Table 1).

The frequencies of *VDR* promoter polymorphisms

Two polymorphisms in the 1a promoter region of the *VDR* gene, −1521 G/C and −1012 A/G, were genotyped successfully in both 188 PCOS women and 143 control women. A summary of the distributions of the genotypes and the allelic frequencies of the *VDR* promoter polymorphisms is presented in Table 2. These two adjacent SNPs, the 1521 bp (G/C) and 1012 bp (A/G), of the human *VDR* gene promoter, were in complete linkage disequilibrium ($r^2 = 1$). The homozygous genotype frequencies for 1521GG/1012AA in normal control and PCOS groups were 91.6% and 96.3%, respectively, while the heterozygous 1521CG/1012GA genotype frequencies in normal control and PCOS groups were 8.4% and 3.7%, respectively. The frequencies of the homozygous 1521G/1012A genotype were slightly increased in the PCOS group (OR = 2.37; 95% CI = 0.91−6.18; $p = 0.071$) as compared with the control group (Table 2). When the PCOS women were further classified into overweight/obese (BMI ≥ 24 kg/m^2) or lean groups (BMI < 24 kg/m^2), it was found that the genotypic distribution of the *VDR 1a* promoter SNPs between these two PCOS groups was similar ($p = 1.000$; Table 2).

Table 1
Descriptive characteristics of the study participants.

	Control group	PCOS group	*p*	PCOS group with BMI <24	PCOS group with BMI ≥24	*p*
Participants (*n*)	143	188		111	75	
Age (y)	31.83 ± 0.48	28.43 ± 0.38	<0.0001*	28.05 ± 0.49	28.97 ± 0.62	<0.0001**
Body weight (kg)	56.48 ± 0.87	61.62 ± 1.06	0.0002*	52.52 ± 0.59	75.08 ± 1.44	<0.0001**
Body height (cm)	159.93 ± 0.47	159.50 ± 0.38	0.4758	159.40 ± 0.47	159.66 ± 0.65	0.7351
BMI (kg/m^2)	22.06 ± 0.32	24.17 ± 0.39	<0.0001*	20.66 ± 0.21	29.37 ± 0.46	<0.0001**
Waist circumference (cm)	79.82 ± 1.79	83.40 ± 1.10	0.1247	75.60 ± 0.93	92.72 ± 1.31	<0.0001**
Systolic blood pressure (mmHg)	114.03 ± 1.23	118.34 ± 1.09	0.0105*	112.60 ± 1.26	126.32 ± 1.46	<0.0001**
Diastolic blood pressure (mmHg)	69.44 ± 1.10	70.45 ± 0.85	0.4671	66.30 ± 0.99	76.22 ± 1.19	<0.0001**
Total cholesterol (mg/dL)	179.46 ± 3.96	179.97 ± 2.85	0.9247	174.80 ± 3.73	186.84 ± 4.32	0.0916
Total triglyceride (mg/dL)	82.61 ± 5.12	102.06 ± 4.47	0.0161*	84.26 ± 5.01	125.70 ± 7.05	<0.0001**
HDL-C (mg/dL)	58.55 ± 2.48	55.378 ± 1.15	0.1919	59.95 ± 1.60	49.42 ± 1.29	<0.0001**
LDL-C (mg/dL)	107.75 ± 4.19	105.64 ± 2.41	0.6611	100.46 ± 3.17	112.26 ± 3.58	0.0477**
Fasting insulin (μU/mL)	6.41 ± 1.15	8.94 ± 0.63	0.0423*	5.83 ± 0.61	13.62 ± 1.08	<0.0001**
2 h insulin (μU/mL)	26.69 ± 1.90	61.87 ± 4.65	<0.0001*	46.19 ± 4.14	85.06 ± 9.11	<0.0001**
HbA1C (%)	5.35 ± 0.03	5.70 ± 0.25	0.3876	5.76 ± 0.42	5.61 ± 0.04	0.6435
Fasting glucose (mg/dL)	86.47 ± 0.69	87.33 ± 0.57	0.3851	85.35 ± 0.68	90.40 ± 0.88	<0.0001**
2 h glucose (mg/dL)	94.75 ± 2.73	112.06 ± 2.39	<0.0001*	105.28 ± 2.89	121.50 ± 3.94	<0.0001**
HOMA index (mg/L)	1.36 ± 0.23	1.97 ± 0.14	0.0256*	1.24 ± 0.13	3.07 ± 0.25	<0.0001**
Fasting sugar/insulin	26.57 ± 3.42	19.57 ± 1.66	0.0415*	26.59 ± 2.58	9.18 ± 0.66	<0.0001**
QUICKI index (mg/L)	3.78 ± 0.27	3.32 ± 0.15	0.1218	3.56 ± 0.26	2.96 ± 0.03	0.0503
LH (mIU/mL)	6.49 ± 0.81	8.26 ± 0.39	0.0299*	9.31 ± 0.57	6.77 ± 0.48	0.0043**
FSH (mIU/mL)	6.58 ± 0.30	6.03 ± 0.15	0.0710	6.22 ± 0.19	5.82 ± 0.26	0.1424
E_2 (pg/mL)	38.04 ± 1.94	45.64 ± 1.78	0.0086*	45.17 ± 2.26	46.23 ± 2.96	0.0327**
TT (nmol/L)	1.30 ± 0.10	1.67 ± 0.06	0.0004*	1.60 ± 0.08	1.83 ± 0.09	0.0002**
SHBG (nmol/L)	57.19 ± 4.41	38.17 ± 2.35	0.0001*	46.23 ± 3.25	25.40 ± 2.48	<0.0001**
17-OHP (ng/mL)	3.60 ± 2.13	1.43 ± 0.09	0.0891	1.37 ± 0.10	1.52 ± 0.16	0.2348
TSH (μU/mL)	1.92 ± 0.12	2.04 ± 0.09	0.4799	1.83 ± 0.10	2.35 ± 0.18	0.0215**
PRL (ng/mL)	12.82 ± 0.70	12.17 ± 0.56	0.4817	12.53 ± 0.70	11.21 ± 0.86	0.3330
25-hydroxyvitamin D (nmol/L)	98.47 ± 3.70	96.42 ± 2.99	0.6637	97.25 ± 3.80	95.59 ± 4.92	0.8952

*$p < 0.05$ by two-sample *t* test;** $p < 0.05$ compared between PCOS with BMI <24, PCOS with BMI ≥24, and control groups by one-way analysis of variance.
17-OHP = 17-OH-progesterone; BMI = body mass index; E_2 = estradiol; FSH = follicle-stimulating hormone; HDL-C = high-density lipoprotein-cholesterol; HOMA = homeostasis model analysis; LDL-C = low-density lipoprotein-cholesterol; LH = luteinizing hormone; PRL = prolactin; QUICKI = quantitative insulin-sensitivity check index; SHBG = sex-hormone binding globulin; TSH = thyroid-stimulating hormone; TT = total testosterone.

Serum concentration of 25-hydroxyvitamin D

The serum concentrations of 25-hydroxyvitamin D in PCOS ($n = 188$, 96.42 ± 2.99 nmol/L) were lower than in the control group ($n = 143$, 98.47 ± 3.70 nmol/L; Table 1), although no statistically significant difference was found between them. When PCOS women were divided into two BMI subgroups, PCOS women with BMI ≥24 kg/m^2 had lower serum concentrations of 25-hydroxyvitamin D (95.59 ± 4.92 nmol/L; $n = 75$) compared with those women having BMI < 24 kg/m^2 (97.25 ± 3.80 nmol/L; $n = 111$; Table 1). We also evaluated the 25-hydroxyvitamin D concentrations between homozygous ($n = 239$) and heterozygous ($n = 19$) carriers of the *VDR 1a* promoter SNPs and found that heterozygous participants showed a significantly low 25-hydroxyvitamin D concentration (63.93 ± 6.10 nmol/L) than homozygous women (100.03 ± 2.40 nmol/L; $p < 0.01$). The findings were consistent in both PCOS and normal control groups (Fig. 1). In control women, 25-hydroxyvitamin D concentration was 102.43 ± 2.39 nmol/L in homozygous individuals ($n = 107$) versus 63.17 ± 6.82 nmol/L in heterozygotes ($n = 12$; $p < 0.01$). In PCOS patients, 25-hydroxyvitamin D concentration was 98.08 ± 3.03 nmol/L in homozygotes ($n = 132$) versus 65.22 ± 12.51 nmol/L in heterozygous women ($n = 7$; $p < 0.05$). However, there was no significant difference in

Table 2
Genotypic distribution of polymorphisms in the *VDR* 1521 and 1012 genes among the control group and the PCOS subgroups according to BMI.

	Control group	PCOS group	OR (95% CI)	*p*	PCOS group with BMI <24	PCOS group with BMI ≥24	*p*
VDR 1521/1012	$n = 143$	$n = 188$			$n = 111$	$n = 75$	
Heterozygous (GC/CT)	12 (8.4%)	7 (3.7%)	1		4 (3.6%)	3 (4.0%)	
Homozygous (CC/TT)	131 (91.6%)	181 (96.3%)	2.37 (0.91–6.18)	0.071	107 (96.4%)	72 (96.0%)	1.000

The *p* value was calculated by logistic regression.
BMI = body mass index; OR = odds ratio; PCOS = polycystic ovary syndrome; VDR = vitamin D receptor.

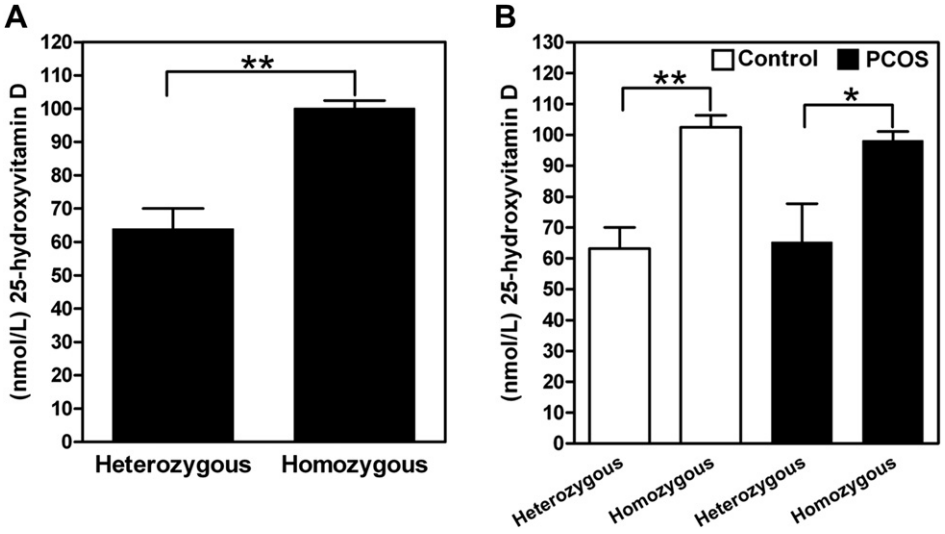

Fig. 1. The difference in serum 25-hydroxyvitamin D concentration between homozygous and heterozygous participants. *$p < 0.05$; **$p < 0.01$.

25-hydroxyvitamin D concentration between PCOS patients and control women, whether homozygous or heterozygous.

There was a significant increase in the serum concentration of 25-hydroxyvitamin D in PCOS patients after receiving metformin therapy 3 months later ($n = 139$; Fig. 2). The 25-hydroxyvitamin D level in PCOS women was increased from pretreated 96.42 ± 2.99 nmol/L to posttreated 104.71 ± 2.71 nmol/L ($p < 0.01$) with 95% confidence interval −9.52 to −7.062. It was interesting that there was no improvement of 25-hydroxyvitamin D level after 3 months of metformin therapy in heterozygous carriers of *VDR 1a* promoter SNPs of PCOS patients, even with original insufficient vitamin D status (pretreated 66.00 ± 13.68 nmol/L vs. posttreated 64.37 ± 7.43 nmol/L; $n = 7$). Only homozygotes demonstrated significantly increased 25-hydroxyvitamin D concentration (pretreated 96.37 ± 2.89 nmol/L vs. posttreated 105.45 ± 2.61 nmol/L; $n = 132$; $p < 0.01$), which was within normal vitamin D status originally, after 3 months metformin therapy.

Discussion

In the present study, we found significantly low serum 25-hydroxyvitamin D levels in heterozygous haplotype carriers of the *VDR 1a* promoter region gene polymorphisms in both PCOS and control groups. Moreover, we observed that metformin treatment was only effective to increase serum 25-hydroxyvitamin D levels in PCOS patients carrying the homozygous haplotype. However, no significant association was found between −1521 G/C and −1012 A/G polymorphisms in the 1a promoter region of the *VDR* gene and PCOS. Besides, we observed that these two SNPs were in complete linkage disequilibrium ($r^2 = 1$). When we further conducted subgroup analyses according to BMI, no significant differences in the genotype distribution in the PCOS group were revealed.

Our study did not find significant association between two polymorphisms in the 1a promoter region of the *VDR* gene and PCOS. One possible explanation is that a high frequency

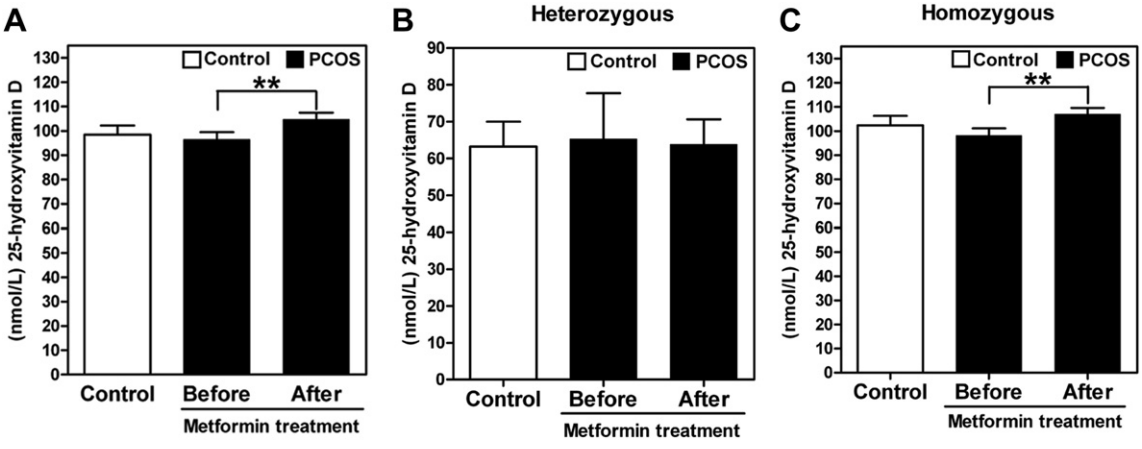

Fig. 2. The change in the serum concentration of 25-hydroxyvitamin D in PCOS after receiving metformin therapy for 3 months in different SNP genotypes. **$p < 0.01$.

of homozygous 1521G/1012A haplotype (>90%) was observed in our study, which is quite different from that of other populations. Jehan et al reported that 1521G and 1012A are evolutionarily conserved among mammals. Homozygous 1521C/1012G haplotype frequency is found in up to 43% Caucasians [17], and in 17.3% healthy French adolescent girls [16]. However, this homozygous haplotype was not observed in our study population. One the other hand, the frequency of the heterozygous 1521CG/1012GA haplotype (57.3%) in French adolescent girls is much higher than that of normal controls in our study (8.4%) [16]. As there is apparent discrepancy in the genotype frequency in different race and ethnic groups and as many as 96% of the PCOS patients in this series were homozygous for these *VDR* SNPs compared with 91% of the controls, we could speculate that the *VDR* 1521C/1012G alleles are unlikely to play a major role in the development of PCOS, but rather may have some effect in the metformin treatment response on serum 25-hydroxyvitamin D levels in PCOS women.

Because of the wide heterogeneity of symptoms and signs of PCOS, which may be predisposed by multiple genetic and environmental factors, it is hard to evaluate putative functional correlations between *VDR* SNPs and PCOS. The findings reported by d'Alesio et al from transfection data demonstrate that human *VDR* promoter activity is 1.9-fold higher in the homozygous 1521G/1012A haplotype compared with that of the homozygous 1521C/1012G haplotype [16]. Moreover, girls with the homozygous 1521C/1012G haplotype of the *VDR 1a* promoter have lower 25-hydroxyvitamin D and insulin-like growth factor-1 levels associated with shorter adult height. However, we cannot make the same comparison to evaluate the relationship between genotype and phenotypes because no homozygous 1521C/1012G haplotype was observed in our study. Despite this, our study found that heterozygous 1521CG/1012GA women showed a significantly lower 25-hydroxyvitamin D concentration than homozygous 1521G/1012A participants in both PCOS and normal control groups, which is coherent with the finding of d'Alesio et al. However, no significant difference in 25-hydroxyvitamin D concentration was found between PCOS patients and normal controls in either homozygous or heterozygous participants, respectively, which might be due to the small sample size.

Vitamin D deficiency is highly prevalent and associated with multiple metabolic risk factors in PCOS patients [20]. The use of metformin may prove beneficial in women with PCOS in metabolic protection and ovulation induction [21]. Elevated insulin levels on a 2-hour 75-g glucose tolerance test is an important parameter to consider in the decision to initiate metformin therapy in women with PCOS. In our previous ultrasound study, metformin treatment improved uterine and ovarian blood flow in women with PCOS [22]. Although there was no difference in the level of 25-hydroxyvitamin D between PCOS and normal control in our present study, we found a significant increase of serum concentration of 25-hydroxyvitamin D in PCOS after receiving metformin therapy for 3 months. Moreover, the 1521C/1012G homozygous participants demonstrated significantly increased 25-hydroxyvitamin D concentration after metformin treatment. The exact pathophysiological mechanisms between 25-hydroxyvitamin D concentration and metformin therapy remains unclear; however, previous studies have reported a positive relationship between total serum calcium and insulin levels as well as insulin resistance [23].

In conclusion, to the best of our knowledge, this is the first study to report the *VDR 1a* promoter gene polymorphism and metformin treatment response on serum 25-hydroxyvitamin D levels in PCOS women. This study found significantly low serum 25-hydroxyvitamin D levels in heterozygous haplotype carriers of *VDR* gene polymorphisms in both PCOS and control groups. Moreover, we also observed that metformin treatment was only effective to increase serum 25-hydroxyvitamin D levels in PCOS patients carrying the homozygous haplotype. A potential limitation of the study is the small sample size; therefore, we have to be cautious in making this conclusion. Further studies using larger sample sizes are warranted to confirm the current findings.

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