Polymorphic variants in vitamin D signaling pathway genes and the risk of endometriosis-associated infertility

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Abstract. It has recently been reported that vitamin D blood plasma levels are associated with reduced risk of endometriosis. The present study aimed to investigate whether the vitamin D binding protein (*GC*), vitamin D receptor (*VDR*), and retinoid X receptor (*RXR*) gene variants may be genetic risk factors for endometriosis-associated infertility. The subjects consisted of 154 women with endometriosis-associated infertility and 347 controls. Using polymerase chain reaction restriction fragment length polymorphism and high resolution melt techniques, the *GC* rs1155563, rs2298849 and rs7041; *RXRA* rs10881578, rs10776909 and rs749759; *VDR BsmI* rs1544410; and *FokI* rs2228570 single nucleotide polymorphisms (SNPs) were investigated in the patients with endometriosis and the healthy controls. The results indicated that no significant differences were observed between the genotype and allele frequencies of all experimental SNPs in the vitamin D signaling pathway genes in women with endometriosis-associated infertility and controls. However, a significant association was present between the A-T haplotype, consisting of *VDR* rs1544410 and rs222857 minor alleles, and endometriosis-associated infertility [OR=1.659 (1.122-2.453), P=0.011]. The results of the present study suggested that *VDR* gene variants act as genetic risk factors for endometriosis-associated infertility.

Introduction

Endometriosis is a gynecological disorder that affects ~10% of women of reproductive age (1). The disease prevalence increases to 30-40% among infertile women (2,3). The development and persistence of endometriosis is associated with alterations in the immune and endocrine systems (3,4). The exact cause of endometriosis and accompanied infertility remains elusive (3,4). Development of endometriosis is hypothesized to be associated with genetic predisposition factors (5). The heritable traits of endometriosis have previously been reported, with a 5-7-fold increased risk of endometriosis development in first-degree relatives (6) There are numerous recognized endometriosis susceptibility genes, which are associated with steroid hormone action, immune response, oxidative stress, glucose homeostasis, vascular and tissue remodeling, and apoptosis (7,8). Endometriosis is characterized by the abnormal survival and growth of endometrial tissue in the abdominal organs (1), which are resistant to apoptosis (8,9). Among the critical factors affecting apoptosis in endometriosis, it has been proposed that survivinl may inhibit apoptosis (10). A recent study reported that increased blood plasma levels of vitamin D are associated with reduced risk of endometriosis, indicating that vitamin D has a beneficial role in the disease (11). Vitamin D is able to inhibit cell proliferation and trigger apoptosis of various types of cancer cell in animal models, as well as *in vitro* (12). Furthermore, the effects of vitamin D on malignant cells is mediated by inhibition of survivin overexpression (13).

Several proteins mediate the biological effects of vitamin D, 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], in humans. Among these are vitamin D binding protein (VDBP), vitamin D receptor (VDR), and retinoid X receptor (RXR) (14-16).

VDBP is encoded by the *GC* gene and is a protein involved in the blood transport of vitamin D and its metabolites (14). VDR binds to RXR, and these heterodimers interact with DNA to induce vitamin D gene expression in target cells (15,16).

A previous study demonstrated that *GC* single nucleotide polymorphisms (SNP) may affect blood plasma vitamin D levels (17). The *VDR* gene also exists in certain genetic variants, which may modulate the biological effect of vitamin D in target cells (18). Furthermore, *RXRA* gene variants are associated with the development of certain tumor types (19). In order to investigate whether *RXRA, GC* and *VDR* gene variants may be genetic risk factors of endometriosis-associated infertility, eight SNPs of these genes located in various blocks of linkage disequilibrium (LD) were selected for the present study.

Materials and methods

Patients and controls. Peripheral blood samples from women with endometriosis and healthy women were obtained from

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the Gynecologic and Obstetrical University Hospital, Division of Reproduction at Poznań University of Medical Sciences (Poznań, Poland). A total of 154 subjects were primary infertile women with endometriosis, and 347 women were used as fertile controls (Table I). The stage of endometriosis was assessed according to the revised classification of the American Society for Reproductive Medicine (20). The inclusion and exclusion criteria for the women with endometriosis and the fertile control women have been previously described (21). The fertile women assigned to the control group were examined for the cause of chronic pelvic pain in the absence of any pelvic abnormalities, as determined by laparoscopy. The controls were diagnosed as having varicose veins in the pelvic floor but no signs of past or present inflammation. Patients and controls were matched by age and were all Caucasian of Polish descent (Table I). Written informed consent was obtained from all participating individuals, and the present study was approved by the Local Ethical Committee of the Poznań University of Medical Sciences.

Table I. Clinical characteristics of women with endometriosis and controls.

Characteristic	Endometriosis	Controls
Number	154	347
Age (years)	33 (20-42)[a]	33 (19-39)[a]
Parity	NA	1 (1-4)[a]
Duration of infertility (years)	3 (1-7)[a]	NA
rASRM (stage)		
Stage I (n=85)	NA	NA
Stage II (n=69)	NA	NA

[a]Median range; rASRM, revised American Society for Reproductive Medicine classification; NA, not applicable.

Genotyping. Genomic DNA was obtained from peripheral blood leucocytes by salt extraction, as previously described (22). The DNA samples were subsequently genotyped for the eight SNPs in the *RXRA*, *GC* and *VDR* genes (Table II). The SNPs were selected with the use of the genome browsers of the International HapMap Consortium (http://www.hapmap.org/index.html.en), the UCSC genome browser (http://genome.ucsc.edu), and the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). The SNPs were selected based on functional significance, distinct location in the LD blocks, and minor allele frequency (>0.1) in the Caucasian population. Genotyping of the *GC* rs1155563 and rs2298849 and *RXRA* rs10881578 and rs10776909 SNPs was conducted by high resolution melting (HRM) using a LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany), with 5X HOT FIREPol® EvaGreen® quantitative polymerase chain reaction (PCR) Mix (Solis BioDyne, Tartu, Estonia). Genotyping of the *GC* rs7041, *VDR Bsm*I rs1544410, *Fok*I rs2228570, and *RXRA* rs749759, SNPs were performed by PCR followed by appropriate restriction enzyme digestion (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with PCR-restriction fragment length polymorphism (PCR-RFLP) according to the manufacturer's instructions (Thermo Fisher Scientific). The primer sequences and thermocycling conditions of the HRM and PCR-RFLP are presented in Table II. Genotyping quality was evaluated by repeated genotyping of 15% randomly selected samples.

Statistical analysis. For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed by Pearson's goodness-of-fit χ^2 statistical test. The differences in allele and genotype frequencies between the patients and the controls were determined using standard χ^2 or Fisher tests. The odds ratio (OR) and associated 95% confidence intervals (95% CI) were also calculated. The data were analyzed under recessive and dominant inheritance models. For the additive inheritance model, the SNPs were tested for association with endometriosis using the Cochran-Armitage trend test. To adjust for the multiple testing, a Bonferroni correction was used. Haplotype based association analysis was performed using the UNPHASED 3.1.5 program (http://sourceforge.net/projects/unphased/files/unphased-3.1.5.zip/download) with the following analysis options: All window sizes, full model and uncertain haplotype (23). The P-values for the global and individual tests of haplotype distribution between cases and controls were determined. Statistical significance was assessed using 1,000-fold permutation testing. P<0.05 was considered to indicate a statistically significant difference.

High order gene-gene interactions among all tested polymorphic loci were investigated using a multifactor dimensionality reduction (MDR) approach with MDR version 2.0 β5 (http://www.epistasis.org/). A detailed explanation on the MDR method has been described previously (24). Based on the obtained testing balanced accuracy and cross-validation consistency values, the best statistical gene-gene interaction models were established. A 1,000-fold permutation test was used to assess the statistical significance of the MDR models (MDR permutation testing module 0.4.9α).

Results

Prevalence of the GC, RXRA and VDR SNPs in women with endometriosis-associated infertility. The distribution of the *RXRA, GC* and *VDR* genotypes did not display deviation from the HWE between patients and control groups (P>0.05). The number of genotypes, OR, and 95% CI evaluation for the eight *GC, RXRA* and *VDR* SNPs are shown in Table III. No association was reported between the *GC, RXRA* and *VDR* SNPs of either the dominant or recessive inheritance models and endometriosis-associated infertility. The statistical significance of the multiple testing determined by correction of SNPs was P=0.00625. The lowest P-values of the trend test were demonstrated for *VDR Fok*I rs2228570 and *RXRA* rs749759 in women with endometriosis-associated infertility (P_{trend}=0.044 and P_{trend}=0.076, respectively).

Association of GC, RXRA, and VDR haplotypes with endometriosis-associated infertility. Haplotype analysis of the *GC, RXRA* and *VDR* SNPs is presented in Table IV. A significant association was demonstrated to exist between the A-T *VDR* (rs1544410 and rs2228570) haplotype and endometri-

Table II. Characteristics of the polymorphisms genotyped in the vitamin D associated genes, and genotyping conditions.

Gene	Chr.	rs no.	SNP function	Alleles[a]	MAF[b]	PCR primers (5'-3')	Annealing temp. (˚C)	PCR product length (bp)	Melt. temp. range (˚C)	HRM	RFLP RE/RFL (bp)
GC	4q13.3	rs7041	missense (p.Asp432Glu)	G/T	0.42	F: GGAGGTGAGTTTATGGAACAGC R: GGCATTAAGCTGGTATGAGGTC	66.3	493		*Hae*III	T=493 G=414+79
		rs1155563	intron	C/T	0.25	F: GGTTATTCTAAGACTGTGCTCTTGC R: ATGTGTTCTCACTGTTCGACTCC	63.0	116	71-78		
		rs2298849	intron	C/T	0.19	F: TCCACTGGCAAAACACATTAC R: GGGACATCTGCATTTATCCTG	60.6	118	73-83		
RXRA	9q34.2	rs10881578	intron	A/G	0.29	F: TCTTGAGCAATGCCAGCAG R: CCACAGCTCACACATCCAATC	60.6	75	80-90		
		rs10776909	intron	C/T	0.21	F: CAGCCTGTGGCCTGCTCA R: AACCTCCGGCCCTTGGAG	60.6	95	82-92		
		rs749759	intron	A/G	0.24	F: ATAGGGCTTGCCTGCCTAGA R: CTCCACCATAGCCCAAGTGA	62.6	382		*Bst*XI	A=382 G=243+139
VDR	12q13.11	rs1544410	intron	A/G B/b	0.40	F: GGAGACACAGATAAGGAAATAC R: CCGCAAGAAACCTCAAATAACA	60.6	248		*Fsp*I	A (B)=248 G (b)=175+73
		rs2228570	missense (p.Met51Thr)	C/T F/f	0.40	F: GCACTGACTCTGGCTCTGAC R: ACCCTCCTGCTCCTGTGGCT	72.5	341		*Fok*I	C (F)=341 T (f)=282+59

[a]According to the single nucleotide polymorphism database. Underline denotes the minor allele. [b]MAF from the 1,000 genomes project for EUR samples. RE, restriction enzyme. RFL, restriction fragment length; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; HRM, high resolution melting; RFLP, restriction fragment length polymorphism.

Table III. Association of polymorphic variants of vitamin D-associated genes and risk of endometriosis.

Gene	rs no.	Alleles[a]	Genotypes cases[b]	Genotypes controls[b]	P_{trend} value	$P_{genotypic}$ value	$P_{allelic}$ value	$OR_{dominant}$ (95% CI)[c]; P-value	$OR_{recessive}$ (95% CI)[d]; P-value
GC	rs7041	G/T	32/73/49	49/184/114	0.242	0.164	0.255	1.048 (0.698-1.574); 0.820	1.595 (0.974-2.611); 0.062
	rs1155563	C/T	15/69/69	25/142/180	0.128	0.314	0.133	1.312 (0.896-1.922); 0.163	1.400 (0.716-2.738); 0.324
	rs2298849	C/T	5/55/93	14/106/226	0.491	0.487	0.494	1.215 (0.820-1.800); 0.331	0.801 (0.283-2.266); 0.675
RXRA	rs10881578	A/G	10/66/78	33/147/166	0.358	0.520	0.362	0.899 (0.615-1.314); 0.581	0.659 (0.316-1.373); 0.262
	rs10776909	C/T	2/51/101	14/114/219	0.328	0.272	0.339	0.898 (0.603-1.336); 0.595	0.313 (0.070-1.395); 0.166[e]
	rs749759	A/G	2/57/95	15/141/191	0.076	0.132	0.094	0.760 (0.516-1.121); 0.166	0.291 (0.066-1.290); 0.109[e]
VDR	rs1544410	A (B)/G (b)	22/76/56	45/154/147	0.263	0.437	0.262	1.293 (0.874-1.912); 0.198	1.115 (0.644-1.931); 0.698
	rs2228570	C (F)/T (f)	37/88/29	65/189/92	0.044	0.122	0.058	1.561 (0.977-2.496); 0.061	1.367 (0.865-2.161); 0.180

[a]Underline denotes the minor allele in the control samples. [b]Order of genotypes: dd/Dd/DD (d is the minor allele in the control samples). [c]Dominant model: dd+Dd, vs. DD (d is the minor allele). [d]Recessive model: dd vs. Dd+DD (d is the minor allele). [e]Fisher exact test. GC, vitamin D binding protein; RXRA, retinoid X receptor A; VDR, vitamin D receptor.

osis-associated infertility [OR=1.659 (1.122-2.453), P=0.011]. However, no other associations between *VDR* haplotypes and endometriosis were demonstrated. Furthermore, there was no association between SNP *GC* and *RXRA* and increased risk of endometriosis-associated infertility. The empirical 5% quantile of the best P-value following 1,000 permutations was 0.01487 for *GC*, 0.01273 for *RXRA* and 0.02565 for *VDR* haplotypes.

Analysis of gene-gene interactions between the GC, RXRA and VDR polymorphisms. Exhaustive MDR analysis evaluating 2-4 loci combinations of all investigated SNPs for each comparison did not indicate statistical significance in predicting susceptibility to endometriosis-associated infertility (Table V). The best combination of possibly interactive polymorphisms was observed for *GC* rs7041 and rs2298849, and *VDR* rs2228570 (testing balanced accuracy, 0.496; cross validation consistency, 70%; permutation test P=0.895).

Discussion

The role of vitamin D in maintaining calcium and phosphorus homeostasis and bone health has been well-established (18,25). However, numerous studies have demonstrated the contribution of vitamin D to several other aspects of health, including human reproduction (26). Vitamin D regulates the expression of numerous genes, including genes associated with steroidogenesis of sex hormones in female reproductive tissues, which also extends to estradiol and progesterone (26,27). Previous studies on humans and animals demonstrated that low vitamin D levels are associated with reduced fertility, poor *in vitro* fertilization outcome, and polycystic ovary syndrome (27,28). In addition, the predicted plasma 25 (OH) D_3 levels have been observed to be inversely associated with endometriosis (27,11).

The role of vitamin D in the development and progression of endometriosis has also been extensively investigated in animal models. Abbas *et al* (29) reported the regression of endometriotic implants treated with vitamin D3 in a rat model (29). Recently, Yildirim *et al* (30) demonstrated the regression of endometriosis in rats treated with vitamin D, as well as associated changes in vascular endothelial growth factor, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-2 expression levels (29).

Polymorphisms located in genes encoding proteins mediated by vitamin D may be risk factors for endometriosis and infertility. The results of the present study demonstrated that *GC, RXRA* and *VDR* SNPs were not separate risk factors for endometriosis-associated infertility. A previous study reported that no association was observed between *Fok*I and *Bms*I *VDR* polymorphisms and endometriosis and/or infertility in Brazilian women (31). However, in the present genetic study, the A-T *Bms*I/*Fok*I *VDR* haplotype was a significant risk factor for endometriosis-associated infertility.

The function of *Bsm*I and *Fok*I SNPs on the biological effects of VDR have been extensively studied (18,32-34). The *Bsm*I polymorphism may change the length of the polyadenylate sequence in VDR transcript (18). Recently, Luo *et al* (32) demonstrated that the *Bsm*I polymorphism

Table IV. Haplotype analysis of SNPs genotyped in the *GC*, *RXRA* and *VDR* genes.

Gene	SNP combination	χ^2	Global P-value[a]	Haplotype	Patients (frequency)	Controls (frequency)	OR (95%CI)	P-value[b]
GC[c]	rs7041_rs1155563	3.022	0.388	G-T	162 (0.53)	396 (0.57)	Reference	
				T-C	91 (0.30)	179 (0.26)	1.243 (0.910-1.697)	0.171
				T-T	45 (0.15)	102 (0.15)	1.078 (0.726-1.602)	0.708
				G-C	8 (0.02)	11 (0.02)	1.778 (0.702-4.502)	0.219
	rs1155563_rs2298849	4.305	0.230	T-T	154 (0.51)	376 (0.55)	Reference	
				C-T	86 (0.28)	178 (0.26)	1.180 (0.858-1.622)	0.309
				T-C	51 (0.17)	121 (0.17)	1.029 (0.706-1.501)	0.882
				C-C	13 (0.04)	13 (0.02)	2.442 (1.106-5.388)	0.023
	rs7041_rs1155563_rs2298849	5.182	0.521	G-T-T	119 (0.39)	295 (0.43)	Reference	
				T-C-T	78 (0.26)	165 (0.24)	1.172 (0.831-1.652)	0.365
				G-T-C	42 (0.14)	97 (0.14)	1.073 (0.705-1.634)	0.741
				T-T-T	35 (0.11)	76 (0.11)	1.142 (0.725-1.797)	0.567
				T-T-C	9 (0.03)	23 (0.04)	0.970 (0.436-2.158)	0.941
				T-C-C	13 (0.04)	13 (0.02)	2.479 (1.116-5.505)	0.022
				G-C-T	8 (0.03)	11 (0.02)	1.803 (0.707- 4.595)	0.211
RXRA[d]	rs10881578_rs10776909	1.348	0.718	A-C	202 (0.66)	423 (0.62)	Reference	
				G-C	51 (0.17)	124 (0.18)	0.861 (0.597-1.243)	0.424
				G-T	35 (0.11)	87 (0.13)	0.842 (0.550-1.291)	0.431
				A-T	20 (0.06)	52 (0.07)	0.805 (0.468-1.385)	0.433
	rs10776909_rs749759	2.812	0.422	C-G	229 (0.74)	486 (0.70)	Reference	
				T-A	37 (0.12)	108 (0.16)	0.727 (0.485-1.090)	0.122
				C-A	24 (0.08)	60 (0.09)	0.849 (0.516-1.398)	0.519
				T-G	18 (0.06)	34 (0.05)	1.124 (0.621-2.032)	0.700
	rs10881578_rs10776909_rs749759	7.923	0.339	A-C-G	176 (0.57)	370 (0.54)	Reference	
				G-C-G	52 (0.17)	112 (0.16)	0.976 (0.671-1.420)	0.899
				G-T-A	17 (0.055)	59 (0.09)	0.606 (0.343-1.070)	0.082
				A-C-A	24 (0.08)	47 (0.07)	1.074 (0.636-1.812)	0.791
				A-T-A	20 (0.06)	46 (0.07)	0.914 (0.525-1.592)	0.751
				G-T-G	17 (0.055)	27 (0.04)	1.324 (0.703-2.493)	0.384
				G-C-A	0 (0.00)	11 (0.02)	0.091 (0.005-1.559)	0.020[f]
				A-T-G	2 (0.01)	8 (0.01)	0.526 (0.110-2.502)	0.514[f]
VDR[e]	rs1544410_rs2228570	6.837	0.077	G-C	94 (0.305)	227 (0.33)	Reference	
				G-T	94 (0.305)	217 (0.32)	1.046 (0.744-1.471)	0.796
				A-C	52 (0.17)	145 (0.21)	0.866 (0.582-1.289)	0.478
				A-T	68 (0.22)	99 (0.14)	1.659 (1.122-2.453)	0.011

The most common haplotype was used as the reference. [a]Likelihood ratio statistic. [b]χ^2 analysis. [c]Empirical 5% quantile of the best P-value: 0.01487. [d]Empirical 5% quantile of the best p-value: 0.01273. [e]Empirical 5% quantile of the best P-value: 0.02565. [f]Fisher exact test. OR, odds ratio; GC, vitamin D binding protein; RXRA, retinoid X receptor A; VDR, vitamin D receptor; SNP, single nucleotide polymorphism.

Table V. Results of gene-gene interactions, as determined by the multifactor dimensionality reduction method.

Genes and rs numbers	Testing balanced accuracy	Cross validation consistency	P-value[a]
*RXRA*_rs749759, *VDR*_rs2228570	0.478	30%	0.977
*GC*_rs7041, *GC*_rs2298849, *VDR*_rs2228570	0.496	70%	0.895
*RXRA*_rs749759, *GC*_rs7041, *GC*_rs2298849, *VDR*_rs2228570	0.496	50%	0.895

[a]Significance of accuracy, empirical P-value based on 1,000 permutations. GC, vitamin D binding protein; RXRA, retinoid X receptor A; VDR, vitamin D receptor.

significantly reduced the mRNA expression levels of VDR in carriers of the A (B) allele, as compared with subjects carrying the GG (bb) genotype (32). The FokI SNP gives rise to two protein forms: A long VDR, encoded by the minor allele form (ATG; f), which contains three additional amino acids, and a shorter form, encoded by (ACG; F) (33). The longer form exhibits 1.7x less efficiency than the shorter form (33). Furthermore, high frequency of the VDR (ATG) (f) allele was associated with a decrease in Th1 immune response (17). A previous study on the role of the *Fok*I SNP demonstrated the presence of increased vitamin D levels in carriers of the TT (ff) genotype, as compared with carriers of the CC (FF) genotype (34).

The presence of the VDR in normal endometrium and endometriotic implants has been demonstrated (35). Furthermore, women suffering from endometriosis express higher levels of VDRs in their endometrial tissue (35,36). In addition, VDRs have been detected in female reproductive tissues, including the ovary, uterus and placenta (37). The expression levels of VDR may also have an important role in the development of endometriosis. Mariani *et al* (38) demonstrated that the VDR agonist elocalcitol exerts protective effects on the implantation and organization of transferred endometriotic implants in murine model of endometriosis.

In conclusion, the present study demonstrated that the A-T (B-f) *VDR* haplotype may be a risk factor for endometriosis-associated infertility. However, in order to further validate the role of this haplotype in endometriosis-associated infertility, similar studies must be conducted in independent ethnicities and in women with idiopathic infertility.

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