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FokI vitamin D receptor polymorphism as a protective factor in intrahepatic cholestasis of pregnancy

Hubert Wolski[1, 2], Grazyna Kurzawinska[1, 3], Marcin Ozarowski[4], Krzysztof Drews[1], Magdalena Barlik[1], Krzysztof Piatek[1], Zbyszko Malewski[1], Aleksandra E. Mrozikiewicz[5], Justyna Magielda-Stola[1], Dorota Kolanowska[1], Marlena Wolek[6], Agnieszka Seremak-Mrozikiewicz[1, 3, 7]

[1]Division of Perinatology and Women's Disease, Poznan University of Medical Sciences, Poznan, Poland
[2]Division of Obstetrics and Gynecology, Poviat Hospital, Zakopane, Poland
[3]Laboratory of Molecular Biology in Division of Perinatology and Women's Diseases, Poznan University of Medical Sciences, Poznan, Poland
[4]Department of Biotechnology, Institute of Natural Fibres and Medicinal Plants, Poznan, Poland
[5]Division of Infertility and Reproductive Endocrinology, Poznan University of Medical Sciences, Poznan, Poland
[6]Department for Research on Stem Cells and Regenerative Medicine, Institute of Natural Fibres and Medicinal Plants, Plewiska/Poznan, Poland
[7]Department of Pharmacology and Phytochemistry, Institute of Natural Fibres and Medicinal Plants, Plewiska/Poznan, Poland

ABSTRACT

Objectives: Intrahepatic cholestasis in pregnancy (ICP) is a pregnancy-specific liver disorder. Its etiology is not fully understood. Increasing evidence indicates the important role of vitamin D and the vitamin D receptor (VDR) in this disorder. The presence of polymorphic variants in the VDR gene could influence its activity and susceptibility to ICP development. The goal of the study was to investigate the role of four genetic polymorphisms of the VDR gene — *Fok* (rs731236), *Bsm* (rs1544410), *Apa* (rs7975232), and *Taq* (rs731236) — in the etiology of ICP in Polish women.

Material and methods: Ninety-eight women with confirmed ICP and 215 healthy pregnant women as a control group were recruited to the study. We examined four SNPs of the *VDR* gene: *BsmI* (rs7975232), *TaqI* (rs1544410), *ApaI* (rs228570), *FokI* (rs731236). Genotyping was performed using the PCR/RFLP method.

Results: We observed higher frequency (borderline significant) of the Ff-ff genotypes containing at least one mutated allele of the *VDR FokI* polymorphism in the control group compared to the ICP group (p = 0.045, OR = 1.71, 95% CI 1.01–2.88). The frequency of the mutated f allele was slightly higher in controls (49.1%) than in the ICP group (43.4%) (OR = 1.26, 95% CI 0.90–1.77), but the difference was not statistically significant (p = 0.196).

Conclusions: Our results showed that the maternal *VDR FokI* polymorphism could play a protective role in ICP development and probably modulate the risk of ICP occurrence in pregnant women in the Polish population. In the future, to confirm these observations, research in larger, ethnically stratified and clinically analyzed groups is necessary.

Key words: intrahepatic cholestasis in pregnancy; vitamin D receptor; genetic polymorphism

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP), affecting 0.2–2% of pregnancies, is a pregnancy-specific liver disorder that typically presents in the third trimester. It is characterized by pruritus associated with elevated serum bile acids and/or aminotransferase levels. The etiology of ICP is complex and still not fully understood. Evidence suggests that it is caused by a combination of hormonal changes, genetic variations, environmental factors and nutritional deficiencies [1]. Also seasonal variation in the frequency of ICP has been observed with a higher prevalence noted in winter months in Scandinavia, Chile and Portugal [2, 3].

Vitamin D (VD) has many important biologic functions, including mineral balance and skeletal maintenance, control of cell proliferation, regulation of differentiation, inhibition of tumor growth and induction of apoptosis [4, 5]. The expression of more than 2000 genes (3% of the human genome) is regulated by the vitamin D signaling pathway [6, 7].

Corresponding author:
Marcin Ozarowski
Department of Biotechnology, Institute of Natural Fibres and Medicinal Plants, 71b Wojska Polskiego St, 60–630 Poznan, Poland
e-mail: mozarowski@poczta.onet.pl

Maternal vitamin D deficiency (VDD) during pregnancy is a global concern that may have important implications for offspring metabolic health. There is increasing evidence that vitamin D plays a role in hepatobiliary homeostasis and in various liver diseases, including ICP [8, 9].

The role of vitamin D in fetal development has been demonstrated, especially in early pregnancy, in skeletal development, and the maturity of the immune system [10, 11]. Vitamin D deficiency has been related to a higher incidence of preeclampsia, fetal hypotrophy, gestational diabetes, preterm labor and bacterial vaginosis in pregnant women [12–16]. Some reports indicate a possible relationship between vitamin D and intrahepatic cholestasis in pregnant women [8, 10]. In addition, it has been proven that VDR is expressed in different parts of the uteroplacental unit and performs different functions in physiological pregnancy. It regulates implantation, affects hormone secretion, and modulates the immunological functions of the placenta, especially in early pregnancy [17, 18].

The vitamin D receptor (VDR), which maps on chromosome 12q12-q14, a member of the nuclear hormone receptor superfamily of ligand-inducible transcription factors, is one of the candidate genes of vitamin D deficiency. Polymorphism of the VDR receptor gene may reduce the response to vitamin D and significantly change the expression of the genes regulated by this vitamin [19]. Currently, more than 200 genes modulated by VDR receptors are known, which indicates the pleiotropic effect of vitamin D. The VDR receptor, activated by 1.25-(OH)D, together with the retinoic acid receptor (RXR) forms a heterodimer that acts as a transcription factor. The VDR-RXR heterodimer binds to the promoter of the selected genes, which enables the initiation/inhibition of the transcription process [20]. Increasing evidence shows the role of vitamin D and the VDR receptor in intrahepatic hemostasis, by inhibiting expression of the cholesterol 7alpha-hydroxylase gene (CYP7A1) and therefore modulating the synthesis of bile acids in hepatocytes, thereby protecting liver cells in humans. One of the possible pathways affecting the modulation of VDR activity and in the same way susceptibility to disease occurrence, is the presence of genetic variants in its gene [21, 22].

Therefore, the purpose of this study was to investigate the effect of four genetic polymorphisms of the *VDR* gene — *Fok* (rs731236), *Bsm* (rs1544410), *Apa* (rs7975232), and *Taq* (rs731236) — on the etiology of ICP in Polish women.

MATERIALS AND METHODS

Subjects

A total of 313 women were recruited to the study: 98 women with confirmed ICP and 215 randomly selected healthy pregnant women who comprised the control group. The research was performed in the years 2013–2017, in two medical centers: the Division of Perinatology and Women's Diseases of Poznan University of Medical Sciences in Poznan and the Department of Gynecology and Obstetrics with Gynecological Oncology Subdivision of Regional Hospital in Zielona Góra. The study was approved by the Poznan University of Medical Sciences Bioethics Committee, Poland, and informed consent was obtained from all the participants. All women included in our study were of Polish ancestry.

In the course of the study the detailed demographic profiles and clinical characteristics were collected from all patients. ICP was diagnosed based on clinical and laboratory criteria: characteristic pruritus without rashes and/or increase in serum bile acids (TBA) ≥ 10 µmol/L in fasting state, increase level of alanine aminotransferase (ALT) ≥ 33 IU/L and aspartate aminotransferase (AST) ≥ 32 IU/L. The women with ICP were observed within 2–3 weeks after delivery when the symptoms had resolved. We excluded women with any causes of hepatic impairment, such as infection with hepatitis viruses (HAV, HBV and HCV), autoimmune diseases, excessive alcohol consumption, HIV infection, biliary obstruction, and other liver and dermatological diseases that cause skin itching. We also excluded those with multiple pregnancies, chromosomal abnormalities, fetal anomalies, maternal infections, pregnancies complicated by thyroid disease, hypertension, and diabetes mellitus, from the study groups. Blood for laboratory tests was secured in all women with ICP before initiating treatment.

Genotyping

We examined four SNPs (rs7975232, rs1544410, rs228570, rs731236) in the *VDR* gene, and all of the SNPs had minor allele frequencies (MAF) greater than 5%. Traditionally these allelic variants have been designated by the upper and lower case of the starting initial of the named loci, *e.g. BsmI* (b and B), *TaqI* (t and T), *ApaI* (a and A) and *FokI* (f and F). Genomic DNA was isolated from whole blood collected in K3-EDTA tubes using the Qiagen DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and was stored at minus 80°C. A NanoDrop 2000 spectrophotometer (Wilmington, DE, USA) was used to evaluate the quality and quantity of DNA. Genotyping was performed in the Molecular Biology Laboratory of Poznan Medical Science University using the PCR/RFLP method as described previously [23–25]. Products were analyzed by electrophoresis on 2.5% agarose gel with Midori Green Advanced DNA Stain (Nippon Genetics, Europe GmbH) (Tab. 1).

Statistical analysis

Statistical analyses were conducted using R version 3.6.0 [26]. For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed using Pearson's goodness-of-fit χ^2 statis-

Table 1. SNPs genotyped in *VDR* gene

Reference SNP ID	rs2228570	rs1544410	rs7975232	rs731236
Traditional Name	FokI	BsmI	ApaI	TaqI
Localization	Exon 2	Intron 8	Intron 8	Exon 9
Allele (Traditional Name Variant)	C/T (F/f)	G/A (b/B)	A/C (A/a)	T/C (T/t)
Amino Acid	Met-Thr	Non-coding	Non-coding	Ile-Ile

Table 2. Clinical, biochemical, obstetrical and perinatal characteristics of the women with ICP and healthy controls

Parameters at mothers	ICP n = 98	Controls n = 215	p
Maternal age [years]	30.40 ± 4.38	30.66 ± 4.66	0.65
Gestational age at delivery [weeks]	36.87 ± 2.73	39.03 ± 1.23	< 0.0001
Systolic blood pressure [mmHg]	110.13 ± 10.71	108.65 ± 10.23	0.26
Diastolic blood pressure [mmHg]	67.57 ± 8.89	66.42 ± 7.83	0.27
Before pregnancy BMI [kg/m^2]	22.93 ± 4.53	21.89 ± 3.59	0.04
Total bile acids [µmol/L]	20.94 ± 27.05	2.80 ± 2.10	< 0.0001
Aspartate aminotransferase [IU/L] AST	255.51 ± 230.50	N.A.	–
Alanine amino transferase [IU/L] ALT	155.33 ± 142.58	N.A.	–
Placenta weight [g]	585.90 ± 154.55	621.18 ± 112.04	0.04
Caesarean section, n (%)	44 (45.83%)	71 (33.02%)	0.03
Primipara, n (%)	43 (44.79%)	83 (38.60%)	0.31
Parameters at neonates			
Neonate birth weight [g]	3094.09 ± 631.30	3418.37 ± 434.21	< 0.0001
Apgar score at 5 min	9.74 ± 0.74	9.97 ± 0.22	< 0.0001
Baby sex (son), n (%)	49 (51.04%)	125 (58.14%)	0.24

N.A. — not accomplished

tic. Continuous variables were expressed as mean ± standard deviation whereas categorical variables were expressed as numbers or percentages. Significant differences between two groups were analyzed by Student's *t*-test. Differences in allele and genotype frequencies between the case and control subjects, odds ratios (ORs) and associated 95% confidence intervals (95% CIs) were evaluated using the SNPassoc package for R [27]. The data were analyzed using codominant, recessive and dominant inheritance models. Haplotype analysis was performed using the Haplo.stats R package [28]. Two-tailed p values less than 0.05 were accepted to be statistically significant.

RESULTS

Demographic and clinical characteristics of subjects

In our study the mean age of cases and controls was 30.40 ± 4.38 and 30.66 ± 4.66 years, respectively (non-significant). ICP women delivered at 36.87 ± 2.73 weeks, whereas controls delivered at term (39.3 ± 1.23 weeks) ($p < 0.0001$). Significant differences in total bile acids (20.94 ± 27.05 vs 2.80 ± 2.10 µmol/L, $p < 0.0001$), neonate birth weight (3094.09 ± 631.30 g vs 3418.37 ± 434.21 g, $p < 0.0001$), and neonate Apgar score (9.74 ± 0.74 vs 9.97 ± 0.22, $p < 0.0001$) were observed. The demographic and clinical characteristics of ICP patients and controls are shown in Table 2.

Association of *VDR* gene polymorphisms with risk of ICP

We explored the *VDR* gene polymorphisms with risk of ICP under codominant, dominant and recessive model, and there were no significant differences in the codominant model (rs2228570, rs1544410, rs797523 and rs731236, all results show $p > 0.05$).

The most interesting observation was the higher frequency of the Ff-ff genotypes containing at least one mutated allele of the VDR FokI polymorphism in the control group compared to the ICP group with the difference of borderline statistical significance ($p = 0.045$, OR = 1.71, 95% CI 1.01–2.88). The frequency of the mutated f allele was slightly higher in controls (49.1%) than in the ICP group (43.4%) (OR = 1.26, 95% CI 0.90–1.77), but the difference was not statistically significant ($p = 0.196$).

For both *VDR BsmI* and *VDR TaqI* polymorphisms a slightly higher frequency of genotypes containing at least one mutated allele in the ICP group was found, but without statistical significance. There was also no contribution of the *VDR ApaI* polymorphism to ICP etiology. Genotype analysis of the *VDR* polymorphisms did not show any significant deviation from Hardy-Weinberg equilibrium in ICP and control groups. The allele and genotype frequencies in ICP women and healthy controls are presented in Table 3.

Haplotype analyses

For four-locus haplotypes six main variants (fbaT, FBAt, fBAt, fbAT, FbaT and FbAT) were constructed for rs2228570,

Table 3. Allele and genotype frequencies of VDR SNPs in ICP women and controls

		ICP n = 98 (%)	Control n = 215 (%)	OR	95%CI	p
rs2228570 (FokI)	CC (FF)	34 (34.7)	51 (23.7)	1.00		
	CT (Ff)	43 (43.9)	117 (54.4)	1.81	1.04–3.17	0.112
	TT (ff)	21 (21.4)	47 (21.9)	1.49	0.76–2.92	
	CC vs CT–TT	64 (65.3)	164 (76.3)	1.71	1.01–2.88	0.045
	CC–TC vs TT	77 (78.6)	168 (78.1)	1.03	0.57–1.83	0.931
Allele	C (F)	111 (56.6)	219 (50.9)	1.00		0.196
	T (f)	85 (43.4)	211 (49.1)	1.26	0.90–1.77	
rs1544410 (BsmI)	GG (bb)	31 (31.6)	91 (42.3)	1.00		
	GA (bB)	48 (49.0)	90 (41.9)	0.64	0.37–1.09	0.191
	AA (BB)	19 (19.4)	34 (15.8)	0.61	0.30–1.22	
	GG vs GA–AA	67 (68.4)	124 (57.7)	0.63	0.38–1.04	0.070
	GG–GA vs AA	79 (80.6)	181 (84.2)	0.78	0.42–1.45	0.439
Allele	G (b)	110 (56.1)	272(63.3)	1.00		0.094
	A (B)	86 (43.9)	158(36.7)	0.74	0.53–1.05	
rs797523 (ApaI)	AA (AA)	26 (26.5)	50 (23.3)	1.00		
	AC (Aa)	51 (52.0)	115 (53.5)	1.17	0.66–2.09	0.811
	CC (aa)	21 (21.4)	50 (23.3)	1.24	0.62–2.48	
	AA vs AC–CC	72 (73.5)	165 (76.7)	1.19	0.69–2.06	0.533
	AA–AC vs CC	77 (78.6)	165 (76.7)	1.11	0.62–1.98	0.719
Allele	A (A)	103 (52.6)	215 (50.0)	1.00		0.605
	C (a)	93 (47.4)	215 (50.0)	1.11	0.79–1.55	
rs731236 (TaqI)	TT (TT)	32 (32.7)	93 (43.3)	1.00		
	TC (Tt)	54 (55.1)	94 (43.7)	0.60	0.36–1.01	0.150
	CC (tt)	12 (12.2)	28 (13.0)	0.80	0.37–1.76	
	TT vs TC–CC	66 (67.3)	122 (56.7)	0.64	0.39–1.05	0.074
	TT–TC vs CC	86 (87.8)	187 (87.0)	1.07	0.52–2.21	0.848
Allele	T (T)	118 (60.2)	280 (65.1)	1.00		0.245
	C (t)	78 (39.8)	150 (34.9)	0.81	0.57–1.15	

rs1544410, rs797523 and rs731236 polymorphisms of the VDR gene. The most frequent variant in ICP patients was FBAt haplotype (28.5%), whereas the estimated prevalence of this haplotype in controls was only 23.9%. When looking at the three-locus haplotypes (*BsmI*, *ApaI*, and *TaqI*), there were also no apparent associations with ICP. Our results indicate that the most common haplotype for the *VDR* gene is baT (47.4% in ICP women and 49.3% in controls). There were no significant differences in the frequency of investigated haplotype variants between ICP women and healthy controls (Tab. 4).

DISCUSSION

Numerous studies have analyzed the influence of vitamin D disturbed metabolism on pregnancy outcome. Several studies analyzed vitamin D levels in groups of women with ICP.

In the Swedish population, reduced VD levels were observed in women with ICP, regardless of the level of bile acids (22 ICP women, 11 healthy pregnant women). In this study statistically significantly lower VD in serum from the ICP group (76.4 ± 23.1 vs 112.0 ± 40 ng/L in controls, p = 0.0041) was observed [8].

Also in the study performed by Gençosmanoğlu Türkmen et al. [9] (40 pregnant women with ICP and 40 healthy pregnant women) vitamin D levels were significantly lower in women with ICP compared to the controls (8.6 ± 4.9 vs 11.3 ± 6.1 ng/mL in controls, p = 0.033). In addition, the authors observed lower VD levels in serum from patients with the severe form of ICP (6.9 ± 2.1 vs 10.3 ± 6.2 ng/mL in severe ICP, p = 0.029) [9].

Although it is known that etiology of ICP is multifactorial, including the genetic basis of the disease, there is not much

Table 4. Haplotype analysis results among SNPs in VDR locus

Haplotype	ICP (freq)	Control (freq)	OR (95% CI)	p value
FBAt	56 (0.285)	103 (0.239)	1.269 (0.867–1.858)	0.218
FbaT	48 (0.244)	93 (0.216)	1.175 (0.789–1.75)	0.426
fbaT	45 (0.229)	119 (0.276)	0.778 (0.525–1.155)	0.213
fBAt	22 (0.112)	43 (0.100)	1.137 (0.66–1.96)	0.641
fbAT	12 (0.061)	42 (0.097)	0.602 (0.309–1.171)	0.131
FbAT	5 (0.025)	14 (0.032)	0.777 (0.276–2.19)	0.633
baT	93 (0.474)	212 (0.493)	0.928 (0.662–1.302)	0.667
BAt	78 (0.397)	146 (0.339)	1.285 (0.907–1.822)	0.157
bAT	17 (0.086)	56 (0.130)	0.634 (0.358–1.122)	0.115
BAT	8 (0.040)	12 (0.027)	1.482 (0.596–3.686)	0.394

haplotypes with frequency < 0.03 are ignored

research focused on this problem. However, there is research demonstrating the importance of *VDR* genetic variants in etiology of primary biliary cirrhosis (PBC) in different populations. The results of these investigations are inconclusive but suggest the possibility of the involvement of genetic variants in the ICP pathomechanism and their influence on liver dysfunction [29-33]. One of them is a meta-analysis of six studies (672 cases and 1148 total controls) showing that the *VDR ApaI* polymorphism is associated with the risk of PBC especially in Asians, while the *VDR TaqI* polymorphism may affect the risk of PBC in Caucasians. However, no significant association was observed between *VDR BsmI* polymorphism and PBC risk [30].

Another meta-analysis of Li et al. [31] (6 studies, 1322 subjects with PBC and 2264 controls) demonstrated that VDR TaqI (rs731236) polymorphism significantly correlated with the risk of PBC (for allele T vs allele t OR = 0.75, p = 0.001; TT + Tt vs tt OR = 0.62, p = 0.005; OR = 0.74, p = 0.016 for recessive model), while for *VDR ApaI* (rs7975232) and *VDR BsmI* (rs1544410) polymorphisms such correlation was not confirmed [31].

The study of 334 PBC patients (195 Japanese and 139 Italians), and 334 healthy sex- and age-matched subjects (179 Japanese and 156 Italians) showed that the BB genotype of *VDR BsmI* polymorphism was significantly associated with PBC risk (OR = 1.80, p = 0.005), both in the Japanese (OR = 13.77, p = 0.001) and Italian (OR = 1.83, p = 0.019) population, but not significantly in the Italian group after Bonferroni correction. The frequency of the *VDR BsmI* polymorphism B allele also was significantly higher in the PBC group (OR = 1.27, p = 0.040), indicating the importance of both BB genotype and the B allele in PBC development [32].

In contrast, a meta-analysis performed by Mo et al. suggests that the *VDR ApaI*, *BsmI*, and *TaqI* polymorphisms do not correlate with increased risk of PBC (*ApaI*: allele A vs allele a OR = 1.132, p = 0.355; BsmI: allele B vs allele b OR = 1.148, p = 0.589; TaqI: allele t vs allele T OR = 1.1432, p = 0.584). Moreover, also in the subgroups separated by ethnicity no relationship was found between the *VDR ApaI*, *BsmI*, and *TaqI* polymorphisms and the occurrence of PBC for the Caucasian or Asian race [33].

As mentioned above, the involvement of *VDR* polymorphisms in ICP etiology is not fully understood, and to the best of our knowledge, this study is the first analysis of this type performed in the population of Polish women. In our study the most interesting result was the higher frequency of genotypes containing at least one mutated allele in the ICP group compared to controls. The frequency of both Ff and ff genotypes was higher in the control group of healthy women with borderline statistical significance (p = 0.045, with OR = 1.71). This observation indicated the protective role of both Ff and ff genotypes of VDR FokI polymorphism in ICP development. This stimulating observation indicated the direction for future research on the mechanism of ICP to prevent the clinical signs of ICP and its serious consequences for the fetus. Considering the haplotypes analysis we have not observed the significance of VDR gene haplotype settings in the etiology of ICP in Polish women. Interestingly, in our population of Polish women the most common haplotype for the *VDR* gene was baT, followed by BAt and bAT, as has been previously described for Caucasians [34].

A limitation of our study is the relatively small number of patients enrolled in the ICP group. On the other hand, these patients were recruited fulfilling the precise criteria for assignment to the study group including the laboratory and clinical signs of ICP. This number of subjects is not sufficient to draw definitive conclusions indicating a direct relationship between VDR polymorphic variants and ICP occurrence but shows the way for further investigations in ICP etiology. Secondly, VDR polymorphisms are unlikely to be the only variants affecting susceptibility to disease and ICP development. No less important is to identify other genetic markers in order to determine the risk groups of

patients predisposed to the development of ICP, as well as to determine the severity of the disease and the possibility of its progression [35–38]. Finally, it is important to note that the exact pathomechanism of the impact of the presence of individual VDR polymorphic variants on ICP etiology remains unexplained. It is known that ICP etiology is multifactorial, with hormonal, genetic and environmental components. Several studies point to the possible role of vitamin D in regulating steroid metabolism, as well as the importance of VDR polymorphisms for maintaining intrahepatic hemostasis. It seems crucial to identify the full ICP pathomechanism that links the presence of VDR genetic variants and serum vitamin D levels to steroid metabolism, bile acid levels, and ICP prevalence. All these findings together could have important clinical implications for our patients and could improve current knowledge about the genetic determinants of ICP.

CONCLUSIONS

To our knowledge this is the first study that suggests an association between maternal VDR FokI variant (rs2228570) and increased risk for ICP in Polish women. This interesting observation noted in relatively small number of patients merit future research to indicate whether this relationship could modulate the ICP development.

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