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Vitamin D receptor polymorphisms in spontaneous preterm birth: a case-control study

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Aim To evaluate the association between the FokI (rs2228570), ApaI (rs7975232), BsmI (rs1544410), TaqI (rs 731236), and Cdx2 (rs11568820) single nucleotide polymorphisms (SNPs) in the vitamin D receptor (*VDR*) gene and spontaneous preterm birth (SPTB), as well as their effect on clinical characteristics of women with SPTB and their newborns.

Methods This case-control study enrolled women who gave birth at the Department of Obstetrics and Gynecology, University Medical Center Ljubljana between 2010 to 2019. Cases were 118 women with spontaneous initiation of PTB after natural conception and 119 controls with a term singleton delivery after an uncomplicated pregnancy. The molecular analysis of *VDR* SNPs employed polymerase chain reaction and restriction fragment length polymorphism.

Results Patients and controls did not significantly differ in the distribution of genotype or allele SNP frequencies. However, the FokI polymorphism had a significant effect on newborn birth weight in women with SPTB but not in controls ($F = 5.17$, $P = 0.007$, one-way ANOVA with *post-hoc* Scheffe test), with newborns of FokI TT carriers having the lowest birth weight ($P = 0.011$). No other *VDR* SNP was associated with any other clinical characteristic of women with SPTB and their newborns.

Conclusion The TT genotype of the VDR FokI polymorphism is associated with newborn birth weight in women of European origin with SPTB.

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Preterm birth (PTB), defined as a birth at less than 37 weeks of gestational age (1), represents the leading determinant of neonatal morbidity and mortality, as well as the main cause of long-term neurologic disabilities in children (2). In spite of the progression in obstetrics and clinical management, PTB incidence in the last decades has not been reduced (3).

PTB can be either spontaneous (SPTB) or medically induced. The heterogeneity of SPTB may be attributed to multiple factors, including socioeconomic status, environmental factors, and genetics (4). The etiology of PTB is still not completely understood, although numerous risk factors have been recognized: previous PTB, uterus and cervix anomalies, repeated miscarriages in the second trimester, *in-vitro* fertilization, multiple pregnancy, vaginal bleeding, abnormal placental factors, low socioeconomic status, smoking, etc (5). Considering that personal and family history of PTB are strongest risk factors for a subsequent PTB, research is focused on identifying variability in potential gene candidates (6,7).

The function of vitamin D metabolism in pregnancy, especially PTB, is currently being intensively investigated (8,9). Vitamin D is a fat-soluble vitamin and hormone precursor, with one of its two forms, cholecalciferol (or vitamin D3), being synthesized in the skin by the exposure to UV radiation. The role of vitamin D in calcium and phosphorus metabolism is well known, but in recent years it has been studied in many other biological processes, particularly in immunomodulation at the feto-maternal interface.

Vitamin D deficiency is related to numerous pregnancy complications (10,11). In addition, several studies clearly showed a causal association between vitamin D status in pregnant women and PTB (12-14). Likewise, preterm infants had lower circulating levels of vitamin D (15).

The association between vitamin D levels and PTB may be mediated by single nucleotide polymorphisms (SNPs) in the vitamin D receptor gene (*VDR*) (16,17). Sixty-three polymorphisms of *VDR* gene have been found, and five of them have been intensively investigated: Cdx2 in the promotion region, FokI in the second coding exon, and BsmI, ApaI, and TaqI in the 3' untranslated region (18). Available evidence indicates a possible causal connection between certain SNPs and PTB, although results are contradictory (16,17,19-21).

Therefore, the aim of our study was to evaluate the association between the FokI (rs2228570), ApaI (rs7975232), BsmI (rs1544410), TaqI (rs 731236), and Cdx2 (rs11568820) SNPs of the *VDR* gene and SPTB, as well as their effect on clinical characteristics of women with SPTB and their newborns.

PATIENTS AND METHODS

Patients

This case-control study enrolled women who gave birth at the Division of Perinatology, Department of Obstetrics and Gynaecology, University Medical Center in Ljubljana, Slovenia between 2010 and 2019. All participants gave written informed consent for study participation. The study was approved by the Slovenian National Medical Ethics Committee and the Ethics Committee for Biomedical Research of the Faculty of Medicine, University of Rijeka.

The patient group comprised 118 women with SPTB. Demographic and clinical data of women with SPTB and their newborns, including family history of SPTB, smoking, maternal age, gestational week of birth, and birth weight of newborns, were collected as described in genetic epidemiology studies on PTB (22) by means of a self-developed interviewer-administered questionnaire. The inclusion criteria for the SPTB group were singleton pregnancy following natural conception and spontaneous initiation of PTB before the 37th week of gestation. Gestational age was estimated from the date of last menstrual period and confirmed by ultrasound in the first trimester. If the measurements differed for more than seven days, gestational age was modified according to the ultrasound measurement. The control group consisted of 119 women who had a term birth of a singleton baby after an uncomplicated pregnancy and were of the same age and parity as patients. The exclusion criteria for both patients and controls were diabetes, hypertension, kidney disease, autoimmune conditions, allergic diseases, birth canal infections, *in vitro* fertilization, and complications of pregnancy and congenital anomalies or evidence of infection in newborns. Cases' and controls' maternal and newborn characteristics are shown in Table 1.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes by standard procedure with Qiagen FlexiGene DNA kit (Qiagen GmbH, Hilden, Germany). All samples were stored at -20 °C. The molecular analysis of *VDR* SNPs employed polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Genotyping

conditions, including primer sequences, PCR protocol, and RFLP fragment sizes are shown in Table 2 (23). PCR reactions were carried out in thermal cyclers (Mastercycle Personal, Eppendorf, Hamburg, Germany and 2720 Thermal Cycler, Applied Biosystems, Carlsbad, CA, USA). Following restriction enzyme digestion, the fragments were separat-

TABLE 1. Characteristics of patients with spontaneous preterm birth (SPTB) and controls

	No. (%) of		
Maternal characteristics	patients*	controls†	*P*
Mean age at delivery (years)‡	30 (18-41)	30 (23-41)	0.385§
Gestational age at delivery		-	
Extremely preterm <28 week	7 (6.2)		
Very preterm 32-28 weeks	33 (29.2)		
Moderate to late preterm 32-37 weeks	73 (64.6)		
Smoking before pregnancy			<0.001¶
yes	37 (32.7)	6 (8.8)	
no	76 (67.3)	62 (91.2)	
Previous PTB		-	
yes	13 (11.5)		
no	100 (88.5)		
Familial PTB		-	
yes	35 (31.8)		
no	75 (68.2)		
Newborn characteristics			
Mean birth weight (g)‡	2334 (605-3915)	3402 (1570-4350)	<0.001§
Congenital anomalies	0	0	
Evidence of infection	0	0	

*available for 113/118 women and newborns with SPTB.
†available for 68/119 control women and newborns.
‡data are presented as mean and range.
§t-test.
¶χ^2 test

TABLE 2. Genotyping conditions for vitamin D receptor single nucleotide polymorphism (*VDR* SNPs)

VDR SNP	Primers	Polymerase chain reaction (PCR) conditions	Fragment sizes (bp)	Restriction enzyme*
ApaI (rs7975232)	5′-GGATCCTAAATGCACGGAGA-3′ 5′-ACGTCTGCAGTGTGTTGGAC-3′	1. 1×: 95 °C (5 min) 35×: 2. 95 °C (60 s) 3. 55 °C (60 s) 4. 72 °C (60 s) 5. 1×: 72 °C (10 min)	PCR product: 265 T allele: 265 G allele: 146 + 119	ApaI
BsmI (rs1544410)	5′-CCTCACTGCCCTTAGCTCTG-3′ 5′-TGCCTCCAAAATCAATCAGG-3′		PCR product: 247 A allele: 247 G allele: 144 + 103	BsmI
Cdx2 (rs11568820)	5′-GGATCCCAAAAGGAAAGGAA-3′ 5′-TGTTCCAGATGGTAAAAATAGAATGA-3′		PCR product: 396 A allele: 316 + 80 G allele: 265 + 80 + 51	HpyCH4III
TaqI (rs731236)	5′-CAGAGCATGGACAGGGAGCAA-3′ 5′-CACTTCGAGCACAAGGGGCGTTAGC-3′		PCR product: 501 T allele: 495 + 6 C allele: 294 + 201 + 6	TaqI
FokI (rs2228570)	5′-AGCTGGCCCTGGCACTGACTCTGCTCT -3′ 5′-ATGGAAACACCTTGCTTCTTCTCCCTC -3′	1. 1×: 94 °C (5 min) 35×: 2. 94 °C (35 s) 3. 61 °C (35 s) 4. 72 °C (1 min) 5. 1×: 72 °C (7 min)	PCR product: 267 C allele: 267 T allele: 195 + 72	FokI

*New England Biolabs (Ipswich, MA, USA).

ed by electrophoresis on 3% agarose gels stained with GelRed™ (Olerup SSP®, Saltsjöbaden, Sweden).

Statistical analysis

Power of the study was calculated with DSS Researcher's Toolkit (https://www.dssresearch.com/resources/calculators/statistical-power-calculator-percentage/), and Hardy-Weinberg equilibrium was calculated with Simple Hardy-Weinberg Calculator – Court Lab (Washington State University College of Veterinary Medicine, Pullman, WA, USA).

The normality of distribution of the numerical variables was tested with the Kolmogorov-Smirnov test. The Pearson chi square test was used to assess the significance of differences in genotype, allele, and haplotype frequencies between the groups. To assess the genetic association with SPTB we calculated odds ratios (OR) and 95% confidence intervals. The *t* test was used to assess the significance of

TABLE 3. Genotype and allele frequencies of vitamin D receptor single nucleotide polymorphism (*VDR* SNPs) in women with SPTB and controls*

		N (%) of					
VDR SNP		women with SPTB	controls	X^2	*P*	OR (95% CI)	*P*
FokI	Genotype						
	CC	41 (34.7)	36 (30.2)	1.23	0.540	reference	
	CT	59 (50)	68 (57.1)			1.31 (0.74-2.31)	0.347
	TT	18 (15.2)	15 (12.6)			0.95 (0.42-2.15)	0.900
	Allele						
	C	141 (59.7)	140 (58.8)	0.01	0.912	reference	
	T	95(40.3)	98 (41.2)			1.04 (0.72-1.50)	0.838
ApaI	Genotype						
	TG	63 (53.4)	63 (52.9)	0.44	0.803	reference	
	GG	30 (25.4)	34 (28.6)			1.13 (0.62-2.07)	0.684
	TT	25 (21.2)	22 (18.5)			0.88 (0.45-1.72)	0.709
	Allele						
	T	113 (47.9)	157 (65.9)	2.03	0.155	reference	
	G	123 (52.1)	131 (55.0)			0.77 (0.54-1.08)	0.131
BsmI	Genotype						
	GG	47 (39.8)	45 (37.8)	0.41	0.813	reference	
	AG	58 (49.1)	63 (52.0)			1.13 (0.66-1.95)	0.788
	AA	13 (11.0)	11 (9.2)			0.88 (0.36-2.17)	0.648
	Allele						
	A	152 (64.4)	153 (64.3)	0.00	0.945	reference	
	G	84 (35.6)	85 (35.7)			1.00 (0.69-1.46)	0.978
TaqI	Genotype						
	TT	53 (44.9)	56 (47.0)	0.84	0.655	reference	
	CT	52 (44.0)	54 (45.4)			0.98 (0.57-1.68)	0.949
	CC	13 (11.0)	9 (7.6)			0.65 (0.26-1.66)	0.372
	Allele						
	T	158 (66.9)	166 (69.7)	0.31	0.578	reference	
	C	78 (33.0)	72 (30.2)			0.88 (0.60-1.29)	0.512
Cdx2	Genotype						
	GG	80 (67.8)	84 (70.6)	0.61	0.737	reference	
	AG	33 (27.9)	32 (23.9)			0.92 (0.52-1.64)	0.786
	AA	5 (4.2)	3 (2.5)			0.57 (0.13-2.47)	0.454
	Allele						
	G	193 (81.8)	200 (84.0)	0.28	0.596	reference	
	A	43 (18.2)	38 (15.9)			0.85 (0.53-1.38)	0.515

*CI – confidence interval; OR – odds ratio; SNP – single nucleotide polymorphism; SPTB – spontaneous preterm birth.

differences in age and newborn birth weight between patients and controls. One-way and two-way analysis of variance (ANOVA) with factorial design and *post-hoc* Scheffe test was used to compare maternal and/or gestational age and fetal birth weight between genotypes of VDR SNPs (one-way ANOVA for individual SNPs, two-way ANOVA for the interaction between SNPs). The level of significance was set at 0.05. Statistical analysis was performed with Statistica for Windows, version 12 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc for Windows, version 14.12.0. (MedCalc Software, Mariakerke, Belgium).

RESULTS

Genetic association of *VDR* SNPs and SPTB

All genotype frequencies in control women were in Hardy-Weinberg equilibrium (data not shown). The statistical power to detect a 1.5-fold increase in the frequency of the FokI (rs2228570) T allele, ApaI (rs7975232) G allele, BsmI (rs1544410) A allele, Cdx2 (rs11568820), A allele, and TaqI (rs731236) C allele was 90%, 99%, 90%, 90%, and 80%, respectively.

No significant differences were found in the genotype distribution of the *VDR* FokI (rs2228570), ApaI (rs7975232), BsmI (rs1544410), TaqI (rs731236), and Cdx2 (rs11568820) genotype or allele frequencies between women with SPTB and controls (Table 3). No SNP was associated with increased odds for SPTB (Table 3).

TABLE 4. Effect of the *VDR* FokI and ApaI gene polymorphisms on newborn birth weight in women with SPTB (two-way ANOVA with factorial design)*

VDR SNP	SS	df	F	*P*
Fok I	3636917	2	4.962	0.009
Apa I	828327	2	1.130	0.327
FokI*ApaI	2490446	4	1.699	0.156

*ANOVA – analysis of variance; SNP – single nucleotide polymorphism; SPTB – spontaneous preterm birth; VDR – vitamin D receptor.

TABLE 5. Effect of the *VDR* FokI genotypes on newborn birth weight in women with SPTB (one-way ANOVA with *post-hoc* Scheffe test)*

FokI genotype	Mean birth weight (g)	*P*
CC	2562.3	0.089†
CT	2273.9	0.312‡
TT	2009.4	0.011§

*ANOVA – analysis of variance; SNP – single nucleotide polymorphism; SPTB – spontaneous preterm birth; VDR – vitamin D receptor.
†CC vs CT.
‡CT vs TT.
§TT vs CC.

Association of clinical characteristics of women and their newborns with *VDR* SNPs

One-way ANOVA showed a significant effect of the FokI and ApaI SNP on newborn birth weight in women with SPTB (FokI F = 5.17, *P* = 0.007; ApaI F = 3.48, *P* = 0.034) but not in the control group (FokI F = 0.57, *P* = 0.569; ApaI F = 0.37, *P* = 0.689). However, two-way ANOVA with factorial design showed no significant interaction between the FokI and ApaI SNPs and found only a significant effect of FokI had on newborn birth weight in women with SPTB (Table 4). *Post-hoc* Scheffe test showed that new-borns of FokI TT carriers had the lowest birth weight (*P* = 0.011) (Table 5). No other *VDR* SNP was associated with any other clinical characteristic of women with SPTB and their newborns.

DISCUSSION

In this study, TT genotype of the *VDR* FokI polymorphism was associated with newborn birth weight women of European origin with SPTB.

Complex molecular and biological interactions between the mother and fetus are not entirely elucidated. Although newborn birth weight is affected by numerous sociological and environmental factors, the strongest effect is exerted by genetics (24). In addition, a causal relationship between pregnancy outcome and vitamin D has been reported (14,25). Although the role of vitamin D in human growth and bone maturation is well-known, vitamin D plays many other roles during gestation, such as influence on implantation, maintenance of normal pregnancy, support of fetal growth through calcium delivery, secretion of numerous placental hormones, and reduction of proinflammatory cytokine production (25). Given the numerous functions of vitamin D, this study focused on the *VDR* gene as a major etiological factor in SPTB. VDR is a mediator of the pleiotropic actions of vitamin D, and its reduced placental expression is implicated in the pathology of complicated pregnancies (26). VDR expression in the human placenta indicates that vitamin D could locally influence the fetal-placental development, growth, and cell differentiation as well as signaling at the maternal-fetal interface.

Although the influence of *VDR* SNPs on VDR protein function and signaling is still largely unclear, some polymorphisms affect the circulating levels of vitamin D and its bio-

logical activity (27). Research suggests that *VDR* genotype affects the optimal vitamin D concentration and reduces the incidence of adverse perinatal outcome. Although the present study showed an effect of the FokI TT genotype on newborn birth weight, we did not find any difference in the frequencies of FokI genotypes and alleles between patients and controls. However, Manzon et al (20) and Javorski et al (17) showed that the maternal FokI T allele increased the risk for SPTB, with an OR of 3.32 and 7.49, respectively. The only previous study that investigated the association between *VDR* SNPs and newborn birth weight found, contrary to our results, that the FokI A allele was associated with lower birth weight (19). We assume that the differences between these studies might be attributable to patient and control selection, sample size, ethnicity, sun exposure, and maternal life-style.

The FokI SNP is considered an independent marker in the *VDR* gene since it does not show linkage disequilibrium with other polymorphisms within the gene. It is a T-C SNP with two protein variants: a long (T-allele) and a short form of VDR protein (C-allele). Variations in the protein sequence significantly affect the protein function. Using a cell line of human fibroblasts, it has been shown that the FokI "f" isoform (T allele) exhibits a less active form of endogenous VDR (28). Since in this study the TT genotype was associated with lower newborn birth weight, we assume that this is the genetic variant responsible for the lower birth weight of prematurely born children. Furthermore, other authors noticed an interesting effect of fetal *VDR* genotype in pregnant women with a lower vitamin D concentration on anthropometric measures at birth. Morley et al (29) observed that infant sequence variants in the *VDR* gene modified the effect of maternal vitamin D deficiency on infant size at birth. Low vitamin D concentrations were associated with lower newborn birth weight only among infants homozygous for the FokI major allele or among heterozygotes.

The function of other *VDR* SNPs – BsmI, ApaI, TaqI, and Cdx2 is less clear. Although we did not demonstrate a significant association with SPTB for any of these SNPs, they represent good research candidates due to the multiple functions of the *VDR* gene, particularly in immunomodulation (cell proliferation, differentiation, invasion, and apoptosis). Baczynska-Strzecha et al (21) showed that individual maternal TaqI, BsmI, and ApaI SNPs did not increase the risk of PTB, but some of the genotype combinations (BsmI-ApaI-TaqI) were significantly more frequent in women with PTB. Rosenfield et al (16) showed the association between both maternal and neonatal *VDR* variants and PTB. The effect of these variants on newborn birth weight was also shown in several studies, which is contrary to our results. Rosenfield et al (16) showed that ApaI AA homozygotes had a 2-fold increased risk for PTB compared with heterozygotes (OR = 1.97). Furthermore, Javorski et al (17) found a significant difference in Cdx2 allele frequencies between the SPTB and control maternal group, and observed an association between this SNP and newborn birth weight. Children of ApaI heterozygotes also had lower birth weight (30). Swamy et al (31) demonstrated the correlation between some other SNPs in the mother's *VDR* gene and lower newborn birth weight in non-Hispanic black women (31). The differences between our and other studies for all five investigated SNPs might be attributed to population differences, participant selection criteria, and sample size.

Future studies should consider environmental factors for low birth-weight, including smoking. The present study showed no association between *VDR* SNP genotypes and smoking before pregnancy. Nevertheless, previous research indicated that cigarette smoke decreased vitamin D production, which could affect *VDR* expression levels (32,33).

A possible limitation of our study is the analysis of only the mother's genotype. However, previous studies have shown that maternal genotype is the most important factor affecting subsequent PTB (34). Additional limitations could be multiple comparisons and possible false positive findings, which is why the robustness of our conclusions should be confirmed by an independent replication study. Our study's advantages are the strict screening of patients with SPTB and exclusion of all PTBs that did not meet the standard clinical definition of SPTB, as well as a sufficient statistical power of the research.

In conclusion, our study showed that the *VDR* FokI SNP was associated with SPTB in women of European origin and that it could affect the lower birth weight of their newborns. Nevertheless, other genetic associations and expression studies in different populations are needed to investigate the role of *VDR* SNPs in SPTB, especially studies assessing maternal vitamin D status.

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Ethical approval given by the Slovenian National Medical Ethics Committee (98/12/10, 2010) and the Ethics Committee for Biomedical Research of the Faculty of Medicine, University of Rijeka, Croatia (2170-29-02/1-19-2).

Declaration of authorship MGK, SO, NP conceived and designed the study; all authors acquired the data; MGK, AB, AP, SO, BP, NP analyzed and interpreted the data; MGK, AB, SO, BP, NP drafted the manuscript; MGK, AB, SO; BP, NP critically revised the manuscript for important intellectual content; all authors gave approval of the version to be submitted; all authors agree to be accountable for all aspects of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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