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Vitamin D Receptor Is a Sepsis-Susceptibility Gene in Chinese Children

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Background: We designed an association study among 267 cases of children with sepsis and 283 healthy controls, by genotyping 9 variants in the *VDR* gene.

Material/Methods: This was a hospital-based, case-control, genetic association study. In addition to 3 genetic modes of inheritance, haplotype and interaction analyses were employed to examine the prediction of *VDR* gene for pediatric sepsis. Effect-size estimates are expressed as odds ratio (OR) and 95% confidence interval (CI).

Results: Two variants in the *VDR* gene, rs2107301 and rs2189480, were found to play a leading role in susceptibility to sepsis in children. The mutant homozygotes of rs2107301 (CC) and rs2189480 (CC) were associated with a reduced risk of sepsis compared with the corresponding wild homozygotes (OR: 0.44 and 0.43, 95% CI: 0.21-0.92 and 0.23-0.81, *p*: 0.03 and 0.009, respectively). The mutations of rs2107301-C and rs2189480-C alleles were associated with reduced sepsis risk. Haplotype C-C-C-C-C-T-C-A-G in the *VDR* gene was significantly associated with a 0.59-fold decreased risk of sepsis (95% CI: 0.12-0.76, *p*: 0.02). In the haplotype–phenotype analysis, significant association was noted for high-density lipoprotein, even after simulation correction (p_{sim} <0.05).

Conclusions: Taken together, our findings indicate that the *VDR* gene may be a sepsis-susceptibility gene in Chinese Han children.

Keywords: Adult Children • MED4 Protein, Human • Risk Assessment • Sepsis

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Background

Sepsis is commonly seen among critically ill children worldwide, with a prevalence rate of 8.2% and an in-hospital mortality rate as high as 25% [1]. The Resolution on Sepsis by the United Nations World Health Assembly in 2017 recognized sepsis as a global threat in children and a priority to address during the next decade [2]. Sepsis is a polygenic and multifactor symptom, with ambiguous etiology [3]. There is evidence from studies of twins with late-onset sepsis [4,5] showing that genetic variability may influence the susceptibility to sepsis through the innate immune system. These genetic variants explain different outcomes of pediatric patients under standardized treatments, and also provide important clues for new mechanisms of sepsis.

The candidate gene approach assumes that the gene with a known biological function is the host one regulating the investigated traits [6,7]. Using this approach, our previous study revealed that the gene encoding vitamin D receptor (VDR) may be a candidate for sepsis risk, as some variants showed a cumulative effect on neonatal sepsis cases [8]. VDR is a kind of nuclear receptor that plays a central role in 1α, 25-dihydroxyvitamin D3's biological actions, and regulates mass gene expression, cellular proliferation and differentiation, and immune response, largely in a ligand-dependent manner [9,10].

Animal experiments have shown that the activation of VDR can protect or attenuate organ injury through inhibiting cell apoptosis, and has even been shown in a mouse model to reverse sepsis-induced immunosuppression through enhancing autophagy [11-13]. Therefore, we developed the hypothesis that the *VDR* gene is a candidate gene of sepsis, and designed a case-control study among 267 children with sepsis and 283 healthy children by genotyping 9 variants in the *VDR* gene to see whether they can predict the risk of sepsis among children in China.

Material and Methods

Study Children

This study was a hospital-based, case-control, genetic association study. Recruitment was carried out at the Emergency Department and Intensive Care Unit (ICU) of the Capital Institute of Pediatrics, Beijing, China. A total of 267 children with sepsis who met the criteria for treatment during the period from October 2017 to April 2020 and 283 healthy controls were included. The conduct of this study was approved by the Ethics Review Committee of the Capital Institute of Pediatrics in Beijing, China (approval ID SHERLL 2013075). All participants read and signed the informed consent form. If children were unable to sign the consent form, the guardians signed on their behalf. This study complied with the Declaration of Helsinki.

Inclusion and Exclusion Criteria

The inclusion criteria were: a) patients under 12 years old admitted to the Pediatric Intensive Care Unit (PICU) diagnosed with sepsis; b) patients were included if they fulfilled criteria of the International Pediatric Sepsis Consensus Conference: Definitions for sepsis and organ dysfunction in pediatrics [14]. The exclusion criteria were as follows: a) patients with known autoimmune disease and cancer; b) using immunosuppressant or immunomodulator; c) congenital organ dysfunction and d) diagnosis of sepsis or shock over 72 h.

DNA Extraction and Quality Control

Venous blood samples were taken in 5-mL Vacutainer tubes with K_3-EDTA from each participant. Plasma was separated by centrifugation at 4°C, then frozen in a freezer at -80°C. The RelaxGene Blood DNA System (Tiangen Biotech, Beijing, China) was used to extract genomic DNA from white blood cells according to the manufacturers' guidelines. The specific process of DNA extraction is provided in **Supplementary File 1**. Then, we used a spectrophotometer to determine concentration (at A260 nm) and purity (at A260/A280 ratio) of DNA.

Variant Selection

Nine variants in the *VDR* gene were selected: rs9729, rs2107301, rs2189480, rs2239185, rs3782905, rs4516035, rs7139166, rs11168266, and rs11168293. The selection of these variants was based on published papers [15-19] and the NCBI-Gene website analysis (https://www.ncbi.nlm.nih.gov/gene/).

Genotyping

The 9 variants in the *VDR* gene were amplified by polymerase chain reaction (PCR) and sequenced by 3730 sequencing analysis. The primer sequences were designed according to the genomic sequence deposited in the NCBI database; 10% of the samples were randomly selected and re-genotyped to ensure the consistency of results. Sequencing results' alignment and multiple comparisons were analyzed by Chromas Lite version 2.01 (http://www.technelysium.com.au). PCR was performed using the following parameters: denaturation for 5 min at 95°C, 30 cycles of 95°C for 15 s, Tm°C for 15 s (annealing temperatures are provided in **Supplementary File 2**), and extension for 1 min at 72°C, with a final extension for 7 min at 72°C. The DNA extraction procedure and primer sequences are provided in **Supplementary File 2**.

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Statistical Analysis

For database management and statistical analysis, we used STATA software Release 14.1 (Stata Corp, TX). Continuous variables are expressed as mean (standard deviation), and compared using the *t* test or Mann-Whitney U test between sepsis and healthy control groups according to its distribution. Categorical variables are described as number (percentage) and we performed the χ^2 test to assess differences between groups. Genotypes and allele differences were compared using the χ^2 test or Fisher's exact test (if 1 observation's frequency was lower than 5). Sepsis risk conferred by different genotypes was calculated by logistic regression analysis after adjusting for age and sex. Effect size was described as odds ratio (OR) and 95% confidence interval (95% CI). Two-sided P<0.05 was considered statistically significant. We used the additive model and dominant model to calculate risk prediction of the 9 studied variants for sepsis risk.

Generally, a haplotype is a combination of multiple alleles on one chromosome. We did some haplotype-based statistical analysis to explore the interactions of these variants. The Haplo.em program was used to compute the haplotype frequencies for the variants in different groups. Haplo.glm and haplo.cc were used to calculate effect sizes for each variant and haplo.score was used to estimate an individual's phenotype as a function of each inferred haplotype. Simulate P (p_{sim}) was the statistical value after 1000 replicates. All the statistical analyses based on haplotype were implemented in the program Haplo.stats software (version 1.4.0) developed using R language (http://www.r-project.org).

Interaction analysis was implemented using the open-source multifactor dimensionality reduction (MDR) software package Release 3.0.2 available from http://www.multifactordimensionalityreduction.org/. Interaction circle graphs were used to visualize the nature of the dependencies.

Results

Baseline Characteristics

Table 1 shows the baseline characteristics of sepsis cases and healthy controls. Controls were significantly older than cases (45 months vs 29 months, $p<0.001$), and males were overrepresented among cases.

Single Variant Analysis

Table 2 shows the genotype distributions and allele frequencies of 9 variants in the *VDR* gene. The genotype distributions differed very significantly for rs2107301 and rs2189480 between cases and controls (p: 0.01 and 0.004, separately). The mutant allele frequencies of rs2107301 (C) and rs2189480 (C) were significantly higher in healthy controls than in sepsis groups (p: 0.003 and 0.001, respectively). There was no hint of significant differences in the other 7 variations, either in genotype distributions or allele frequencies, between the 2 groups. The effect size of each variations' genotype was calculated using their wild homozygous genotype as a reference (**Table 2**). The mutant homozygous genotypes of rs2107301 (CC) and rs2189480 (CC) were associated with reduced risk compared to the wild homozygous genotype (OR: 0.44 and 0.43, 95% CI: 0.21-0.92 and 0.23-0.81, p: 0.03 and 0.009, respectively). Among the genotypes of rs2189480, the CA genotype also showed a lower risk of sepsis than the AA type (OR: 0.62, 95% CI: 0.43-0.90, p: 0.01). In the genotypes of the remaining variations, the differences in effect size were not significant. All p values were calculated after adjusting for age and sex in logistic regression analysis.

Table 1. The baseline characteristics of study children.

Characteristics	Cases	Controls
Sample size	267	283
Age (months)	29±35*	45±38
Sex, N (%)	182 (68%)*	149 (53%)
TG (mmol/L)	1.4 (1.0-2.0)*	0.8 (0.6-1.0)
HDL (mmol/L)	0.7 (0.5-1.0)*	1.4 (1.1-1.6)
LDL (mmol/L)	1.9 (1.3-2.4)	2.2 (1.9-2.7)
AMY (U/L)	26 (10-46)*	55 (37-74)
LD (U/L)	415 (306-746)*	229 (207-253)
HBDH (mmol/L)	290 (215-463)*	169 (150-189)
CKMB (IU/L)	19 (7-31)	22 (18-25)
Urea (mmol/L)	3.4 (2.5-5.1)	3.8 (3.1-4.6)
ALB (g/L)	34 (30-38)*	44 (41-46)
ALP (U/L)	129 (92-177)	196 (158-241)
WBC (10^9/L)	16 (11-23)*	7.2 (5.8-8.6)
RBC (10^9/L)	4.0 (3.5-4.5)*	4.6 (4.3-4.9)
HGB (mmol/L)	110 (95-124)*	126 (121-135)
PLT (mmol/L)	300 (210-428)	296 (236-337)

TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; AMY – amylase; LD – lactate dehydrogenase; HBDH – hydroxybutyrate dehydrogenase; CKMB – creatine phosphokinase-Mb; ALB – albumin; ALP – alkaline phosphatase; WBC – white blood cell; RBC – red blood cell; HGB – hemoglobin; PLT – platelets. Data are expressed as mean±standard deviation or median (interquartile range) or number (%), where appropriate. The p was calculated using the t-test or Mann-Whitney U test or χ^2 test, where appropriate. * $p<0.05$ between cases and controls.

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Table 2. Genotype and allele distributions of *VDR* gene 9 studied variants between cases and controls, and genotype-based risk prediction for sepsis mortality risk.

Variants		Cases (n=267)	Controls (n=283)	*p* Value	OR, 95% CI, *p* value
rs9729	CC	133 (52%)	144 (53%)	0.98	Reference group
	AC	104 (41%)	112 (41%)		1.05, 0.72-1.52, 0.81
	AA	18 (7%)	18 (6%)		1.13, 0.55-2.31, 0.74
	A	27%	27%	0.87	
rs2107301	TT	154 (59%)	135 (48%)	0.01	Reference group
	TC	95 (36%)	121 (43%)		0.70, 0.48-1.01, 0.06
	CC	12 (5%)	26 (9%)		0.44, 0.21-0.92, 0.03
	C	23%	31%	0.003	
rs2189480	AA	137 (52%)	109 (39%)	0.004	Reference group
	CA	106 (40%)	137 (49%)		0.62, 0.43-0.90, 0.01
	CC	19 (8%)	35 (12%)		0.43, 0.23-0.81, 0.009
	C	27%	37%	0.001	
rs2239185	CC	135 (51%)	154 (55%)	0.32	Reference group
	CT	108 (41%)	115 (41%)		1.11, 0.77-1.59, 0.59
	TT	20 (8%)	13 (5%)		1.94, 0.91-4.14, 0.09
	T	28%	25%	0.24	
rs3782905	CC	192 (80%)	195 (75%)	0.20	Reference group
	CG	48 (20%)	60 (23%)		0.83, 0.53-1.31, 0.43
	GG	1 (0.4%)	5 (2%)		0.26, 0.03-2.39, 0.23
	G	11%	13%	0.13	
rs4516035	TT	249 (95%)	267 (96%)	0.41	Reference group
	CT	14 (5%)	10 (4%)		1.62, 0.66-3.94, 0.29
	CC	0 (0%)	1 (0.4%)		Unavailable
	C	3%	2%	0.59	
rs7139166	CC	248 (95%)	271 (96%)	0.54	Reference group
	CG	14 (5%)	11 (4%)		1.52, 0.64-3.63, 0.35
	GG	0 (0%)	1 (0.4%)		Unavailable
	G	3%	2%	0.69	
rs11168266	GG	135 (52%)	149 (53%)	0.72	Reference group
	GA	104 (40%)	105 (38%)		1.15, 0.79-1.68, 0.45
	AA	20 (8%)	26 (9%)		0.89, 0.46-1.70, 0.72
	A	28%	28%		
rs11168293	GG	250 (96.53%)	272 (96.80%)	0.86	Reference group
	GT	9 (3%)	9 (3%)		1.24, 0.46-3.35, 0.68
	TT	0 (0%)	0 (0%)		Unavailable
	T	3%	3%	0.93	

OR – odds ratio; 95% CI – 95% confidence interval. The *p* values were calculated after adjusting for age and sex in a logistic regression analysis.

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Table 3. The unadjusted and adjusted risk prediction of 9 studied variants for sepsis mortality risk under additive and dominant models, respectively.

Variants	Model	Additive model	Dominant model
rs9729	Unadjusted	1.02, 0.78-1.35, 0.87	1.02, 0.72-1.43, 0.93
	Adjusted	1.06, 0.79-1.40, 0.71	1.06, 0.74-1.51, 0.76
rs2107301	Unadjusted	0.66, 0.50-0.87, 0.003	0.64, 0.45-0.90, 0.01
	Adjusted	0.68, 0.51-0.91, 0.008	0.65, 0.46-0.93, 0.02
rs2189480	Unadjusted	0.64, 0.49-0.83, 0.001	0.58, 0.41-0.81, 0.002
	Adjusted	0.64, 0.49-0.84, 0.001	0.58, 0.41-0.83, 0.003
rs2239185	Unadjusted	1.19, 0.90-1.56, 0.23	1.14, 0.82-1.60, 0.44
	Adjusted	1.24, 0.93-1.65, 0.15	1.19, 0.84-1.69, 0.33
rs3782905	Unadjusted	0.74, 0.50-1.09, 0.13	0.77, 0.50-1.17, 0.21
	Adjusted	0.77, 0.51-1.17, 0.22	0.79, 0.51-1.24, 0.31
rs4516035	Unadjusted	1.23, 0.57-2.62, 0.60	1.37, 0.61-3.06, 0.45
	Adjusted	1.34, 0.59-3.01, 0.48	1.49, 0.62-3.55, 0.37
rs7139166	Unadjusted	1.16, 0.55-2.45, 0.70	1.28, 0.58-2.81, 0.55
	Adjusted	1.28, 0.58-2.84, 0.55	1.41, 0.60-3.30, 0.43
rs11168266	Unadjusted	0.99, 0.76-1.28, 0.93	1.05, 0.75-1.47, 0.80
	Adjusted	1.02, 0.78-1.34, 0.87	1.10, 0.77-1.56, 0.60
rs11168293	Unadjusted	1.09, 0.43-2.79, 0.86	1.09, 0.43-2.79, 0.86
	Adjusted	1.24, 0.46-3.35, 0.68	1.24, 0.46-3.35, 0.68

Data are expressed as odds ratio, 95% confidence interval, *p* value. The *p* values were calculated after adjusting for age and sex in a logistic regression analysis.

Table 4. Haplotype frequencies (>1% in all cases and controls) of variants in VDR genes between cases and controls, and haplotype-based risk prediction for sepsis mortality risk.

Haplotype*	All	Cases	Controls	Hap-Score	P	P_{sim}	OR (95% CI, P)
C-T-A-C-C-T-C-G-G	0.44	0.46	0.41	2.16	0.03	0.04	Ref.
A-T-A-T-C-T-C-A-G	0.18	0.20	0.17	1.15	0.25	0.27	1.03 (0.70-1.50, 0.25)
C-C-C-C-G-T-C-G-G	0.10	0.09	0.11	-1.48	0.14	0.15	0.65 (0.41-1.02, 0.14)
C-C-C-C-C-T-C-G-G	0.06	0.05	0.07	-1.21	0.23	0.26	0.60 (0.33-1.12, 0.23)
C-T-C-C-C-T-C-G-G	0.06	0.05	0.06	-0.71	0.48	0.52	0.76 (0.42-1.36, 0.48)
A-C-C-T-C-T-C-A-G	0.04	0.04	0.04	-0.37	0.71	0.65	0.86 (0.41-1.81, 0.71)
C-C-C-C-C-T-C-A-G	0.03	0.01	0.04	-2.32	0.02	0.03	0.31 (0.12-0.76, 0.02)
C-C-A-C-C-T-C-G-G	0.02	0.03	0.02	0.68	0.50	0.54	1.30 (0.51-3.33, 0.50)
A-T-C-T-C-T-C-A-G	0.01	0.01	0.02	-0.87	0.38	0.39	0.53 (0.11-2.62, 0.38)

Hap-Score – haplotype score, P_{sim} – simulated *p* value; OR – odds ratio; 95% CI – 95% confidence interval. * Alleles in each haplotype were in order of rs9729, rs2107301, rs2189480, rs2239185, rs3782905, rs4516035, rs7139166, rs11168266 and rs11168293 polymorphisms.

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Table 5. Global testing of all haplotypes with anthropometric index and clinical biomarkers.

Characteristics	Global statistics	P	P_{sim}
Age (month)	14.05	0.93	0.77
TG (mmol/L)	12.35	0.93	0.77
HDL (mmol/L)	69.56	<0.001	0.02
LDL (mmol/L)	15.12	0.82	0.69
AMY (U/L)	5.92	1.00	0.82
LD (U/L)	6.76	0.99	0.77
HBDH (mmol/L)	9.00	0.99	0.82
CKMB (IU/L)	5.30	1.00	0.75
Urea (mmol/L)	27.26	0.25	0.22
ALB (g/L)	3.98	1.00	0.69
ALP (U/L)	30.94	0.12	0.18
WBC (10^9/L)	10.99	0.98	0.79
RBC (10^9/L)	19.02	0.70	0.60
HGB (mmol/L)	23.01	0.46	0.37
PLT (mmol/L)	27.04	0.25	0.27

TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; AMY – amylase; LD – lactate dehydrogenase; HBDH – hydroxybutyrate dehydrogenase; CKMB – creatine phosphokinase-Mb; ALB – albumin; ALP – alkaline phosphatase; WBC – white blood cell; RBC – red blood cell; HGB – hemoglobin; PLT – platelets.

The unadjusted and adjusted risk predictions of the 9 studied variants in the *VDR* gene for sepsis mortality risk were calculated in both additive and dominant models to correct for some mutant homozygotes that were not sufficiently numerous, which has an impact on the predictive value (**Table 3**). In general, 2 variants, rs2107301 and rs2189480, showed significant protective effects under the additive and dominant model. The effect sizes were: rs2107301 in the additive model (OR: 0.68, 95% CI: 0.51-0.91, *p*: 0.008), rs2107301 in the dominant model (OR: 0.65, 95% CI: 0.46-0.93, *p*: 0.02), rs2189480 in the additive model (OR: 0.64, 95% CI: 0.49-0.84, *p*: 0.001), and rs2189480 in the dominant model (OR: 0.58, 95% CI: 0.41-0.83, *p*: 0.003). All the results were meaningful regardless of whether factors were adjusted or not.

Haplotype Analysis and Haplotype–Phenotype Association

Table 4 presents the derived haplotype frequencies and risk estimates for pediatric sepsis. Haplotype C-T-A-C-C-T-C-G-G (alleles arranged by order of rs9729, rs2107301, rs2189480, rs2239185, rs3782905, rs4516035, rs7139166, rs11168266, and rs11168293, with the same hereafter) was the most common (frequency less than 1% is not displayed). Frequency of haplotype C-C-C-C-C-T-C-A-G was significantly higher in controls than in cases (4% vs 1%, *p*: 0.02) and was significantly associated with a 0.59-fold decreased risk of pediatric sepsis (95% CI: 0.12-0.76, *p*: 0.02). Other haplotypes had no significant association with sepsis risk.

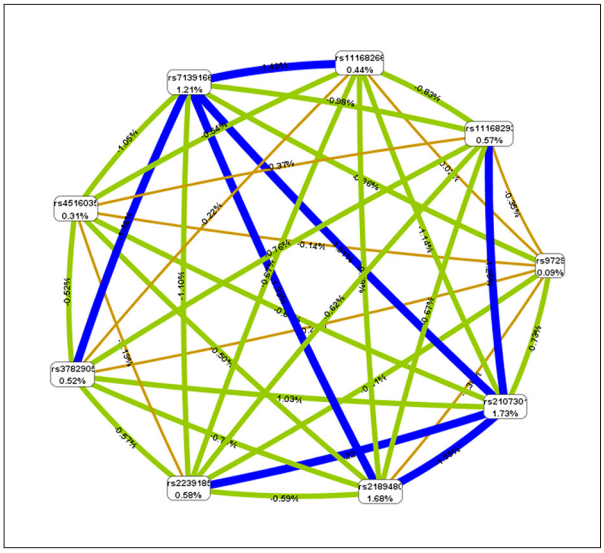

Figure 1. The interaction of 9 variants under study in predisposition to pediatric sepsis.

We explored a haplotype–phenotype association by taking all haplotypes of the 9 studied variants as a whole, and tested the comprehensive correlation of haplotypes with all collected baseline characteristics (**Table 5**). A significant association was noted for high-density lipoprotein (HDL) in sepsis patients after simulation correction (p_{sim}<0.05).

Interaction Analysis

The interaction of the 9 variants under study in predisposition to sepsis in children is displayed in **Figure 1**. Blue lines and green lines represent antagonism, and the intensity of blue was stronger than that of green; red lines or orange lines represent synergistic effect, and the intensity of red was stronger than that of orange (this study did not produce red or orange lines, indicating that antagonism was dominant among various sites). Overall, there was no evidence of synergistic interaction between variants.

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Discussion

Our results support the hypothesis of this study, by showing that the *VDR* gene might be a candidate gene for pediatric sepsis. Specifically, 2 variants in the *VDR* gene, rs2107301 and rs2189480, may play a leading role in susceptibility to sepsis. In addition, the mutations of rs2107301-C and rs2189480-C alleles may be factors protecting against sepsis. To the best of our knowledge, this is the first study reporting an association between these 9 variants and sepsis susceptibility.

The study by Zeljic et al [20] evaluated 4 variants in the *VDR* gene in predisposition to sepsis, and they found only 1 variant was significantly associated with this disease. However, whether the *VDR* gene is a sepsis-susceptibility gene still needs further exploration. To extend the findings of previous studies, we genotyped 9 intronic variants in the *VDR* gene and found the significant contribution of rs2107301 and rs2189480 to sepsis in Chinese children, especially for the mutant homozygotes. As the 2 significant variants are mapped on the intronic regions, it is reasonable to speculate that 1 or 2 of these variants may be in linkage disequilibrium with other functional variants in or adjacent to the *VDR* gene. In addition, the 2 variants might play a part in the process of selective splicing during transcription [21].

Our study provides evidence of associations of rs2107301 and rs2189480 with sepsis risk for the first time. As is known, sepsis is a multi-system disease. While VDR is almost ubiquitously expressed, nearly all cells respond to 1,25-dihydroxyvitamin D [22]. Animal experiments showed that when VDR-deficient mice were exposed to predisposing factors, their sensitivity to autoimmune diseases increased significantly, which may due to inhibiting the NF-κB pathway and activating autophagy [9,22]. After blocking NF-κB pathways, VDR signaling suppressed miR-802 expression or activated CD4+ T cells to participate in the immune response [23]. It is reasonable to consider that *VDR* gene mutation may affect immune status so that pathogens cannot be eliminated effectively, and then cause an outbreak of sepsis. These variants have also been reported to be related with primary diseases such as essential hypertension, as well as tumor development [24-26].

In the test of all haplotypes with clinical biomarkers, we found a significant association noted for HDL in sepsis patients. This suggests that the effect of haplotypes on sepsis is related to HDL. HDL particles are emulsions of metabolites, lipids, and proteins that protect by removing cholesterol from tissues, so high levels of HDL have a protective effect on the body [27-29]. However, a low HDL level was associated with the *VDR* gene [30,31]. Previous studies have shown that *VDR* genetic variants can change energy metabolism by regulating adipose tissue activity, especially in rs2189480, an AAA haplotype can even increase the risk of cardiovascular disease [32-34]. The effect of the *VDR* gene on HDL occurs via multiple channels. Studies in VDR-KO mice and VDR-Tg mice showed that overexpression of *VDR* corresponded with decreased expression of uncoupling proteins [34,35]. However, mice with adipose-specific *VDR* deletion expressed elevated white adipose tissue and overexpressed Ucp1 and Pparg, supporting that these genes act as VDR targets in mature adipocytes [35]. White adipose tissue dysfunction has a serious impact on both the quantity and function of HDL and other lipoproteins, indirectly providing evidence that VDR regulates HDL [36]. Collectively, our research supports the views discussed above, and we speculate that HDL may be a ring node in the pathogenesis of *VDR* genetic variants associated with sepsis risk.

Limitations

Several limitations should be acknowledged for this study. Firstly, there was a significant difference between groups in sex and age. Although we carried out statistical adjustments, the bias cannot be eliminated. Secondly, as an observational case-control association study, our results cannot prove the cause-effect relationship between *VDR* gene and pediatric sepsis risk. Thirdly, a small-sample bias may exist in this study, so the results should be considered as preliminary. Fourthly, the genetic variation coverage in the *VDR* gene was limited.

Conclusions

Taken together, our findings indicate that the *VDR* gene may be a sepsis-susceptibility gene in children from China. In particular, 2 variants in the *VDR* gene, rs2107301 and rs2189480, played a leading role in predicting pediatric sepsis risk. We agree that future large-scale, well-designed studies are warranted to further confirm or refute the findings of this study.

Acknowledgement

We are grateful to all participating patients for their cooperation and willingness.

Ethics Approval and Consent to Participate

Our study design received approval from the Ethics Committee of Children's Hospital Affiliated to the Capital Institute of Pediatrics. Written informed consent was obtained from a parent or guardian for all study participants. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest

None declared.

Statement

The funder of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication

Data Sharing Statement

We are glad to make our research data available upon reasonable request.

Declaration of Figures Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

Supplementary Materials

File 1. DNA extraction procedure.

1. Preparation of samples: Pipet 200 µl sample to the microcentrifuge tube. If the volume is less than 200 µl, adjust volume to 200 µl with buffer GA. If the sample volume is more than 200 µl, e.g.300 µl-1ml, please refer the following step: add 3 times volume Red Cell Lysis Buffer to the sample, then invert the tube and close the cap. Stay the tube in room temperature (15-25°C) for 5min, and centrifuge at 12,000 rpm (~13,400×g) for 1 min, then discard the flow-through and pipet 200 µl buffer GA and mix by pulse-vortexing.

2. Add 20 µl Proteinase K, mix thoroughly by vortexing. If the sample is tissue: incubate at 56°C until the tissue is completely lysed.

3. Add 200 µl Buffer GB to the sample, mix thoroughly by vortexing, and incubate at 70°C for 10 min to yield a homogeneous solution. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

4. Add 200µl ethanol (96-100%) to the sample, and mix thoroughly by vortexing for 15 s. A white precipitate may form on addition of ethanol. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

5. Pipet the mixture from step 4 into the TIANamp Spin Column CB3 (in a 2 ml collection tube) and centrifuge at 12,000 rpm(~13,400×g) for 30s. Discard flow-through and place the spin column into the collection tube.

6. Add 500 µl Buffer GD to TIANamp Spin Column CB3, and centrifuge at 12,000 rpm (~13,400×g) for 30s, then discard the flow-through and place the spin column into the collection tube.

7. Add 700µl Buffer PW to TIANamp Spin Column CB3, and centrifuge at 12,000 rpm (~13,400×g) for 30s. Discard the flow-through and place the spin column into the collection tube.

8. Add 500µl Buffer PW to TIANamp Spin Column CB3, and centrifuge at 12,000 rpm (~13,400×g) for 30s. Discard the flow-through and place the spin column into the collection tube.

9. Centrifuge at 12,000 rpm (~13,400×g) for 2 min to dry the membrane completely.

10. Place the TIANamp Spin Column CB3 in a new clean 1.5 ml microcentrifuge tube, and pipet 50-200 µl Buffer TE or distilled water directly to the center of the membrane. Incubate at room temperature (15-25°C) for 2-5 min, and then centrifuge for 2 min at 12,000 rpm (~13,400×g).

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File 2. Primers involved in the experiment.

Rs number	Primer name	Primer sequences	Tm (°C)	Product length (bp)
rs9729	VDR-P1-F	CCTTGCACCTGCATCCGTAG	60	856
	VDR-P1-R	GAAAAGGACACCGGACCATGA		
rs2107301	VDR-P2-F	CTGTGCCGTTCATTTGGA	60	284
	VDR-P2-R	AGTGTTGGGCTGTCTGGT		
rs2189480	VDR-P3-F	AGAGAGCAGCTGAGGCAATG	60	415
	VDR-P3-R	GGACACCATTACGCTCTGGA		
rs2239185	VDR-P4-F	TCATTGCCATTTCCATAC	60	387
	VDR-P4-R	GACATTTACACCCTCCTCT		
rs3782905	VDR-P5-F	GACAGATGGTCCTTTCTT	58	693
	VDR-P5-R	AATCCACTACCCACTACA		
rs4516035	VDR-P6-F	GATGGCTGCGGAAAACTCAC	60	470
	VDR-P6-R	ATTGAGTTGTGAGGGGCTGG		
rs7139166	VDR-P7-F	AGGCATAGCGTTTGATTG	58	212
	VDR-P7-R	GGTATTGGTGGTTGGAAA		
rs11168266	VDR-P8-F	TTTCACCATAGCAAACCCAA	60	391
	VDR-P8-R	CTCCCAGCAGGCAGACAT		
rs11168293	VDR-P9-F	ACCAAGGAACCCTGAGAC	60	481
	VDR-P9-R	GAAGGCAAATAGGAAACAAT		

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