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Impact of gene polymorphisms involved in the vitamin D metabolic pathway on the susceptibility to and severity of autism spectrum disorder

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This study explores the association between genetic variations in the vitamin D pathway and autism spectrum disorder (ASD) susceptibility and severity in Thai children. A total of 276 participants, including 169 children with ASD and 107 healthy controls, were recruited. Genotyping of vitamin D pathway genes (*CYP2R1*, *CYP27B1*, *GC*, and *VDR*) was conducted using TaqMan-based real-time PCR, while serum vitamin D levels were measured by chemiluminescence immunoassay. ASD severity was assessed via the Childhood Autism Rating Scale, 2nd Edition. Results reveal that the *VDR* gene (ApaI) rs7975232 is linked to a reduced ASD risk. In contrast, the *GC* gene rs7041 (A > C) polymorphism shows a significant association with increased ASD risk and severity, particularly in individuals with both the *GC* gene polymorphism and vitamin D insufficiency. Additionally, there was a higher prevalence of the *GC1s* isoform and *GC1s-GC1s* haplotype in children with ASD, associated with ASD severity. This study identified that individuals possessing *GC* rs7041 *C* alleles and the *GC1s* genotype (rs7041*C*/rs4588*G*) exhibit an increased susceptibility to and more severity of ASD. Further studies with larger cohorts are essential to fully understand these genetic polymorphisms' roles.

Keywords Autism spectrum disorder, Vitamin D gene polymorphisms, Thai Children

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by repetitive behaviors, communication and social interaction deficiencies, and language impairments[1,2]. During the past two decades, the prevalence of ASD has significantly increased both in Thailand and worldwide, impacting the global economy and health in significant ways[3–5]. However, the etiology of ASD remains unknown. Most believe

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that ASD is influenced by several risk factors, including genetic and environmental factors[6]. Previous research explains the link between vitamin D and ASD, and various hypotheses have emerged. The research indicates that vitamin D plays a role in DNA repair, which helps prevent mutations and reduce the risk of ASD[7]. Furthermore, vitamin D plays a vital role in producing antioxidants such as glutathione and superoxide dismutase[8], which help reduce neuroinflammation commonly observed in individuals with ASD[9,10]. Numerous autoimmune phenomena have been documented over decades in individuals with ASD[11]. Vitamin D is known to regulate inflammatory processes and autoimmune responses[12], suggesting it may help reduce both inflammation and autoimmune activity. Additionally, vitamin D stimulates brain cells to produce growth factors, such as nerve growth factor (NGF). Due to its neurotrophic and neuroprotective effects, vitamin D is believed to promote neuronal growth and may help slow the progression of neurodegenerative diseases[13]. Previous studies have shown that a lack of vitamin D increases the risk of ASD and the severity of ASD symptoms[14–16]. Similarly, animal studies have linked vitamin D deficiency to the development of ASD[17]. Identifying risk factors for ASD is crucial in developing new prevention and treatment strategies[18].

Vitamin D is received into the body through synthesis in the skin, depending on UVB skin exposure[19]. The active form of vitamin D, $1,25(OH)_2D$, is produced after two metabolic steps. The main circulating vitamin D metabolite, 25(OH)D, is initially produced in the liver during the first metabolic phase from 7-dehydrocholesterol, primarily catalyzed by the enzyme CYP2R1. Subsequently, the kidney's CYP27B1 enzyme catalyzes the second metabolic step, converting 25(OH)D into $1,25(OH)_2D$[20]. All forms of vitamin D and the vitamin D-binding proteins encoded by the *GC* gene are transported throughout the body and have been detected in human cerebrospinal fluid[21,22]. Vitamin D leads to the stimulation of the expression of its target genes via binding to the vitamin D receptor (VDR), which translocate into the nucleus upon activation[23].

Previous studies have identified associations between single-nucleotide polymorphisms (SNPs) in the vitamin D metabolic pathway and an increased risk of ASD. A study of the Han Chinese population revealed that the genetic variation of gene *CYP27B1* (rs4646536) is significantly associated with an increased risk of ASD[18]. Previous studies showed the relation between the genetic variation of genes in the vitamin D pathway; the *CYP2R1* gene (rs10741657) and the *GC* gene (rs4588, rs7041) are associated with ASD[24,25]. Vitamin D binding protein (VDBP), also known as group-specific component (GC), is highly polymorphic with significant functional implications. Key polymorphisms include rs7041 and rs4588. These polymorphisms lead to the formation of three major GC isoforms : (1) *GC1s* (rs7041*C*-rs4588*G*), (2) *GC1f* (rs7041*A*-rs4588*G*), and (3) *GC2* (rs7041*A*-rs4588*T*), resulting in six different haplotypes: *GC1f-GC1f*, *GC1f-GC1s*, *GC1s-GC1s*, *GC1f-GC2*, *GC1s-GC2*, and *GC2-GC2*. Five common SNPs of *VDR*, which modulate gene transcription, are rs731236 (*Taq1*), rs11568820 (*Cdx2*), rs1544410 (*Bsm1*), rs2228570 (*FokI*) and rs7975232 (*ApaI*). The structure, transcriptional activity, and functions of VDR proteins have been associated with these SNPs[26,27], and these SNPs also have been linked to an increased incidence of ASD in children[28–30].

However, the effect of genetic variation in genes of the vitamin D pathway on ASD has not been elucidated, and there has been no investigation of this aspect in Thai children with ASD. The objective of this study is to investigate the association between genetic variations in the vitamin D pathway and the susceptibility to and severity of ASD in Thai children. The researcher expects that this research could elucidate how to assess the risk and prognosis of ASD in the Thai population more accurately and precisely in the future.

Results

Demographic and clinical characteristics

The demographic and clinical profiles of the participants are presented in Table 1. The study comprised a total of 276 participants, with 169 individuals assigned to the ASD group and 107 individuals to the control group. Among the ASD group, consisting of 169 subjects (141 males and 28 females), the mean age was 7.97 years. In comparison, the control group, comprising 107 subjects (90 males and 17 females), had a mean age of 8.61 years. A statistical analysis revealed no significant differences in age or gender distribution between the two groups (p value = 0.47 and p value = 0.88, respectively). Furthermore, parameters such as the weight, height, body mass index (BMI), and total 25(OH)D levels did not exhibit significant variations between the control and ASD groups (Table 1).

Characteristics of the subjects	Healthy control ($N = 107$)	ASD group ($N = 169$)	p value
Age (years)	8.61 ± 3.89	7.97 ± 4.15	0.47
Gender - Male - Female	90 (84.11%) 17 (15.89%)	141 (83.43%) 28 (16.57%)	0.88
Weight (kg)	33.71 (18.49)	34.69 (21.09)	0.96
Height (cm)	130.59 (22.49)	129.41 (24.15)	0.53
Body Mass Index	18.42 (4.89)	18.93(4.75)	0.40
Total 25(OH)D (ng/ml) (mean ± SD)	20.94 ± 6.95 ($N = 95$) [#]	20.31 ± 6.61 ($N = 146$) [#]	0.89

Table 1. Demographic and clinical characteristics. [#]From the initial 276 participants, those who had used vitamin D supplements or medications affecting vitamin D levels within the past 6 months were excluded, resulting in a final sample size of 241 (95 healthy controls and 146 cases).

The association of Vitamin D pathway gene polymorphisms with the susceptibility to autism spectrum disorder

We explored the potential association between gene polymorphisms in the vitamin D pathway and susceptibility to ASD using the Chi-square test under a dominant genetic model. An association between the risk of ASD and the *GC* gene rs7041 *(A > C)* polymorphism was established. Notably, individuals with the *GC* gene rs7041 (*AC + CC*) genotype exhibited a significantly elevated association with the ASD group compared to those with the *AA* genotype (odds ratio = 1.74, 95% confidence interval = 1.13–2.66, *p* value = 0.01) (Table 2). However, after correcting for false discovery rates (FDR) with the Benjamini-Hochberg method, the association was no longer significant (Supplementary Table 1). Additionally, no significant association was observed between the polymorphisms of the *GC* (rs4588), *CYP27B1* (rs4646536), *CYP2R1* (rs10741657), and V*DR; Taq1* (rs731236), *ApaI* (rs7975232), *BsmI* (rs1544410), *FokI* (rs2228570), and *Cdx2* (rs11568820) genes and susceptibility to ASD. Further research should investigate a larger cohort to confirm the findings and clarify the relationships between the examined gene polymorphisms and ASD susceptibility.

The association of Vitamin D pathway gene polymorphisms with the severity of autism spectrum disorder

In this study, ASD was categorized into two groups: (1) minimal-to-no Symptoms and (2) mild-to-moderate and severe symptoms, based on the scores obtained from the Childhood Autism Rating Scale, 2nd Edition (CARS-2)[31,32]. The minimal-to-no symptoms group comprised 15 children, while the mild-to-moderate and severe symptoms group included 154 children. The findings, based on the Chi-square test under a dominant genetic model, revealed a significant association between individuals carrying the *GC* gene rs7041 (*AC + CC*) genotype and the severity of the mild-to-moderate and severe symptoms group with ASD compared to those with the *AA* genotype (odds ratio = 2.48, 95% confidence interval = 0.99–6.19, *p* value = 0.04) (Table 3). However, after applying FDR adjustment with the Benjamini-Hochberg procedure, the previously significant association between the *GC* gene rs7041 (A > C) variant and the severity of autism spectrum disorder was no longer significant (Supplementary Table 2). Moreover, other gene polymorphisms did not demonstrate significant associations with the severity of childhood ASD (Table 3). Additionally, a separate analysis was conducted to assess the association between genetic variants and the severity of ASD across three groups: minimal-to-no symptoms (*N* = 15), mild-to-moderate symptoms (*N* = 50), and severe symptoms (*N* = 104). However, this analysis did not yield significant results before or after adjusting for FDR using the Benjamini-Hochberg procedure, likely due to the limited sample size (see Supplementary Table 3). Future studies should consider expanding the sample size to enhance the statistical power of the analysis and allow for more robust conclusions regarding the association between genetic variants and ASD severity.

The association of the GC gene (rs7041) and vitamin D insufficiency (vitamin D levels < 30 ng/mL) with the risk of autism spectrum disorder

The investigation of the *GC* gene rs7041 *(A > C)* variant, coupled with vitamin D insufficiency, was significantly associated with the susceptibility to ASD. The reference group consisted of participants with the *A* alleles of rs7041 and sufficient vitamin D levels. The children exhibiting both the *GC* gene rs7041 *C* allele and vitamin D insufficiency demonstrated an elevated risk of ASD compared to the reference group (odds ratio = 3.14, 95% confidence interval = 1.36–7.27, *p* value = 0.01) (Table 4).

Genes	SNP ID	Genetic Variants	Healthy control, *N* (%) (*N* = 107)	ASD group, *N* (%) (*N* = 169)	OR (95%CI)	*p* value
GC	rs4588	*GG* *GT + TT*	64 (59.81%) 43 (40.19%)	94 (55.62%) 75 (44.38%)	1.19 (0.79–1.80)	0.42
GC	rs7041	*AA* *AC + CC*	57 (53.27%) 50 (46.73%)	67 (39.64%) 102 (60.36%)	1.74 (1.13–2.66)	0.01*
CYP27B1	rs4646536	*AA* *AG + GG*	24 (22.43%) 83 (77.57%)	35 (20.71%) 134 (79.29%)	1.10 (0.64–1.93)	0.72
CYP2R1	rs10741657	*AA* *AG + GG*	8 (7.48%) 99 (92.52%)	17 (10.06%) 152 (89.94%)	0.72 (0.31–1.71)	0.46
Taq I	rs731236	*AA* *AG + GG*	85 (79.44%) 22 (20.56%)	145 (85.80%) 24 (14.20%)	0.64 (0.39–1.04)	0.07
Apa I	rs7975232	*CC* *CA + AA*	40 (37.38%) 67 (62.62%)	73 (43.20%) 96 (56.80%)	0.79 (0.50–1.22)	0.29
Bsm I	rs1544410	*CC* *CT + TT*	87 (81.31%) 20 (18.69%)	137 (81.07%) 32 (18.93%)	1.02 (0.63–1.64)	0.95
Fok I	rs2228570	*AA* *AG + GG*	27 (25.23%) 80 (74.77%)	37 (21.89%) 132 (78.11%)	1.20 (0.71–2.05)	0.50
Cdx2	rs11568820	*CC* *CT + TT*	39 (36.45%) 68 (63.55%)	56 (33.14%) 113 (66.86%)	1.16 (0.73–1.80)	0.53

Table 2. The association of Vitamin D pathway gene polymorphisms with the susceptibility to autism spectrum disorder. Statistical significance was assessed using the Chi-square test under a dominant genetic model. * *p* value < 0.05.

Genes	SNP ID	Genotype	CARS2 Classification of Severity of ASD		OR (95% CI)	*p* value
			Minimal-to-no Symptoms (*N* = 15)	Mild-to Moderate and Severe Symptoms (*N* = 154)		
GC	rs4588	*GG* *GT* + *TT*	9 (9.57%) 6 (8.00%)	85 (90.43%) 69 (92.00%)	1.22 (0.49–3.01)	0.67
GC	rs7041	*AA* *AC* + *CC*	9 (13.43%) 6 (5.88%)	58 (86.57%) 96 (94.12%)	2.48 (0.99–6.19)	0.04*
CYP27B1	rs4646536	*AA* *AG* + *GG*	4 (11.43%) 11 (8.21%)	31 (88.57%) 123 (91.79%)	1.44 (0.47–4.46)	0.52
CYP2R1	rs10741657	*AA* *AG* + *GG*	2 (11.76%) 13 (8.55%)	15 (88.24%) 139 (91.45%)	1.43 (0.30–6.58)	0.65
Taq I	rs731236	*AA* *AG* + *GG*	14 (9.66%) 1 (4.17%)	131 (90.34%) 23 (95.83%)	2.46 (0.53–11.23)	0.23
Apa I	rs7975232	*CC* *CA* + *AA*	5 (6.85%) 10 (10.42%)	68 (93.15%) 86 (89.58%)	0.63 (0.22–1.75)	0.37
Bsm I	rs1544410	*CC* *CT* + *TT*	12 (8.76%) 3 (9.38%)	125 (91.24%) 29 (90.62%)	0.93 (0.32–2.59)	0.88
Fok I	rs2228570	*AA* *AG* + *GG*	5 (13.51%) 10 (7.58%)	32 (86.49%) 122 (92.42%)	1.91 (0.66–5.43)	0.22
Cdx2	rs11568820	*CC* *CT* + *TT*	4 (7.14%) 11 (9.73%)	52 (92.86%) 102 (90.27%)	0.71 (0.23–2.16)	0.54

Table 3. The association of Vitamin D pathway gene polymorphisms with the severity of autism spectrum disorder. Statistical significance was assessed using the Chi-square test under a dominant genetic model. * *p* value < 0.05.

GC gene rs7041 (A > C)	Vitamin D insufficient	OR (95%CI)	*p* value
A Allele (*N* = 14)	No	1	
A Allele (*N* = 154)	Yes	1.60 (0.71–3.60)	0.24
C Allele (*N* = 7)	No	1.49 (0.49–4.48)	0.48
C Allele (*N* = 66)	Yes	3.14 (1.36–7.27)	0.01*

Table 4. The association between the *GC* gene (rs7041) and vitamin D insufficiency (vitamin D levels < 30 ng/mL) with the risk of autism spectrum disorder. Statistical significance was calculated by the chi-square test. * *p* value < 0.05. From the initial 276 participants, those who had used vitamin D supplements or medications affecting vitamin D levels within the past 6 months were excluded, resulting in a final sample size of 241.

The association of the GC isoform genotype and haplotype with the susceptibility to autism spectrum disorder

The data reveal a comparison of the distribution of genotype and haplotype of the *GC* gene isoforms (rs7041(*A* > *C*)/rs4588(*G* > *T*)) between two groups: the healthy control group and the ASD group. The genotypes comprise *GC1s* (rs7041*C*-rs4588*G*), *GC1f* (rs7041*A* rs4588*G*), and *GC2* (rs7041*A*-rs4588*T*), while the haplotype represents various combinations of these genotypes. The genotypic analysis indicates that *GC1s* (rs7041*C*-rs4588*G*) is notably more prevalent among children with ASD (34.32%) compared to healthy controls (23.83%), with a statistically significant *p* value of 0.01. Interestingly, the *GC1s-GC1s* haplotype is more prevalent in the ASD cohort (8.28%) than in the control group (0.94%), with a statistically significant *p* value of 0.02. These findings demonstrate the association of the *GC1s* isoform with the risk of ASD in Thai children (Table 5).

The association of the GC isoform genotype and haplotype with the severity of autism spectrum disorder

Table 6 presents an analysis of the association between the GC isoform genotype and haplotype and the severity of autism spectrum disorder. Significantly, the *GC1s* genotype (rs7041C-rs4588*G*) exhibited a higher prevalence among individuals with ASD with mild-to-moderate and severe symptoms (35.71%) compared to those with minimal-to-no symptoms (20.00%), with a *p* value of 0.03. Additionally, upon examining the distribution of isoform haplotypes, the *GC1s-GC1s* haplotype was observed more frequently in individuals with mild-to-moderate and severe symptoms (9.09%) compared to those with minimal-to-no symptoms (0.00%), with a significant *p* value of 0.01. These data highlight the potential impact of the *GC1s* isoform genotype and haplotype on the severity of ASD symptoms, providing valuable insights into the genetic factors associated with ASD. We also conducted a separate analysis of ASD subjects classified into three groups: minimal-to-no symptoms (*N* = 15), mild-to-moderate symptoms (*N* = 50), and severe symptoms (*N* = 104). However, the results were not statistically significant (Supplementary Table 4). Future research should explore larger and more diverse populations to better understand the potential relationships between symptom severity in ASD and genetic factors.

Isoform[#]	Genotype (rs7041(A > C)/ rs4588(G > T))	Healthy control, *N* (%)	ASD group, *N* (%)	*p* value
GC1s	*C/G*	51 (23.83%)	116 (34.32%)	0.01*
GC1f	*A/G*	117 (54.67%)	144 (42.60%)	
GC2	*A/T*	46 (21.50%)	78 (23.08%)	
Isoform haplotype[##]				
GC1f-GC1f	*A/G-A/G*	29 (27.10%)	34 (20.12%)	0.02*
GC1f-GC1s	*A/G-C/G*	34 (31.78%)	46 (27.22%)	
GC1s-GC1s	*C/G-C/G*	1 (0.94%)	14 (8.28%)	
GC1f-GC2	*A/G-A/T*	25 (23.36%)	30 (17.75%)	
GC1s-GC2	*C/G-A/T*	15 (14.02%)	42 (24.85%)	
GC2-GC2	*A/T-A/T*	3 (2.80%)	3 (1.78%)	

Table 5. The association of the GC isoform genotype and haplotype with the susceptibility to autism spectrum disorder. N: absolute number of genotype/isoform haplotype, %: genotype/isoform haplotype frequency. Statistical significance was calculated by the chi-square test. * p value < 0.05. Healthy control ($N = 107$), ASD group ($N = 169$). [#]*GC1s* (rs7041*C*/rs4588*G*), *GC1f* (rs7041*A*/rs4588*G*) and *GC2* (rs7041*A*/rs4588*T*). [##]*GC1f-GC1f* (rs7041*AA*/rs4588*GG*), *GC1f-GC1s* (rs7041*AC*/rs4588*GG*), *GC1s-GC1s* (rs7041*CC*/rs4588*GG*), *GC1f-GC2* (rs7041*AA*/rs4588*GT*), *GC1s-GC2* (rs7041*AC*/rs4588*GT*) and *GC2-GC2* (rs7041*AA*/rs4588*TT*).

Isoform[#]	Genotype (rs7041(A > C)/ rs4588(G > T))	CARS2 Classification of Severity of ASD		*p* value
		Minimal-to-no Symptoms, *N* (%)	Mild-to Moderate and Severe Symptoms, *N* (%)	
GC1s	*C/G*	6 (20.00%)	110 (35.71%)	0.03*
GC1f	*A/G*	18 (60.00%)	126 (40.91%)	
GC2	*A/T*	6 (20.00%)	72 (23.38%)	
Isoform haplotype[##]				
GC1f-GC1f	*A/G-A/G*	5 (33.33%)	29 (18.83%)	0.01*
GC1f-GC1s	*A/G-C/G*	4 (26.67%)	42 (27.27%)	
GC1s-GC1s	*C/G-C/G*	0 (0.00%)	14 (9.09%)	
GC1f-GC2	*A/G-A/T*	4 (26.67%)	26 (16.88%)	
GC1s-GC2	*C/G-A/T*	2 (13.33%)	40 (25.98%)	
GC2-GC2	*A/T-A/T*	0 (0.0%)	3 (1.95%)	

Table 6. The association of the GC isoform genotype and haplotype with the severity of autism spectrum disorder. N: absolute number of genotype/isoform haplotype, %: genotype/isoform haplotype frequency. Statistical significance was calculated by the chi-square test. * p value < 0.05. Minimal-to-no Symptoms ($N = 15$), Mild-to Moderate and Severe Symptoms ($N = 154$). [#]*GC1s* (rs7041*C*/rs4588*G*), *GC1f* (rs7041*A*/rs4588*G*) and *GC2* (rs7041*A*/rs4588*T*). [##]*GC1f-GC1f* (rs7041*AA*/rs4588*GG*), *GC1f-GC1s* (rs7041*AC*/rs4588*GG*), *GC1s-GC1s* (rs7041*CC*/rs4588*GG*), *GC1f-GC2* (rs7041*AA*/rs4588*GT*), *GC1s-GC2* (rs7041*AC*/rs4588*GT*) and *GC2-GC2* (rs7041*AA*/rs4588*TT*).

Multivariate analysis of predictive factors for the susceptibility to autism spectrum disorder

Table 7 presents a multivariate analysis of predictive factors for susceptibility to autism spectrum disorder, including nine polymorphisms—*GC* rs4588, *GC* rs7041, *CYP27B1* rs4646536, *CYP2R1* rs10741657, *TaqI* rs731236, *ApaI* rs7975232, *BsmI* rs1544410, *FokI* rs2228570, and *Cdx2* rs11568820 as well as the vitamin D levels. The final multivariate model showed that the *GC* rs7041 polymorphism was significantly associated with an increased risk of ASD, with an odds ratio of 1.72 (95% confidence interval = 1.11–2.67, p value = 0.01). Additionally, the *VDR (ApaI)* rs7975232 polymorphism was significantly associated with a reduced risk of ASD, with an odds ratio of 0.68 (95% confidence interval = 0.48–0.96, p value = 0.03) after multivariate adjustment. The results indicate that the *GC* rs7041 polymorphism and the *VDR* (*ApaI*) rs7975232 polymorphism are independent predictors of susceptibility to autism spectrum disorder in the Thai population.

Multivariate analysis of predictive factors for the severity of autism spectrum disorder

Table 8 presents a multivariate analysis of predictive factors for the severity of autism spectrum disorder, including nine polymorphisms—*GC* rs4588, *GC* rs7041, *CYP27B1* rs4646536, *CYP2R1* rs10741657, *TaqI* rs731236, *ApaI* rs7975232, *BsmI* rs1544410, *FokI* rs2228570, and *Cdx2* rs11568820 as well as the vitamin D levels. The final multivariate model showed that the *GC* rs7041 polymorphism was significantly associated with the severity of

Predictive factors	SNPs	OR (95% CI)	*p* value
GC	rs7041 (*A* > *C*)	1.72 (1.11–2.67)	0.01*
VDR (ApaI)	rs7975232 (*C* > *A*)	0.68 (0.48–0.96)	0.03*

Table 7. Multivariate analysis of predictive factors for the susceptibility to autism spectrum disorder ($N = 241$). Data are from logistic regression analyses: Forward stepwise method. Variables entered into the method: *GC* rs4588, *CYP27B1* rs4646536, *CYP2R1* rs10741657, *TaqI* rs731236, *BsmI* rs1544410, *FokI* rs2228570, *Cdx2* rs11568820 and vitamin D levels. *p value < 0.05. From the initial 276 participants, those who had used vitamin D supplements or medications affecting vitamin D levels within the past 6 months were excluded, resulting in a final sample size of 241.

Predictive factors	SNPs	OR (95% CI)	*p* value
GC	rs7041 (*A* > *C*)	3.39 (1.04–11.03)	0.01*

Table 8. Multivariate analysis of predictive factors for the severity of autism spectrum disorder ($N = 146$). Data are from logistic regression analyses: Forward stepwise method. Variables entered into the method: *GC* rs4588, *CYP27B1* rs4646536, *CYP2R1* rs10741657, *TaqI* rs731236, *BsmI* rs1544410, *FokI* rs2228570, *Cdx2* rs11568820 and vitamin D levels. * p value < 0.05. From the initial 169 ASD, those who had used vitamin D supplements or medications affecting vitamin D levels within the past 6 months were excluded, resulting in a final sample size of 146.

ASD, with an odds ratio of 3.39 (95% confidence interval = 1.04–11.03, p value = 0.01). The results suggest that the *GC* rs7041 polymorphism is an independent predictor of the severity of ASD in the Thai population.

Discussion

Vitamin D has been identified as a significant environmental factor associated with the onset of ASD[33]. Numerous studies have indicated that vitamin D is involved in immune modulation, anti-inflammatory processes, cell proliferation and differentiation, as well as neurodevelopment and protection[12,34], which might be related to the susceptibility and severity of ASD[9–11,13].

To our knowledge, this is the first study to assess the impact of both polymorphisms in the vitamin D pathway on the susceptibility to and severity of ASD in the Thai population. The findings showed no significant associations between the genetic variants *GC* rs4588, *CYP27B1* rs4646536, *CYP2R1* rs10741657, *Taq I* rs731236, *Bsm I* rs1544410, *Fok I* rs2228570, and *Cdx2* rs11568820, and the risk or severity of ASD. However, this study suggests that the *VDR* gene polymorphisms rs 7975232 (ApaI) is significantly linked to a decreased risk of ASD. Moreover, our study revealed notable associations between the *C* allele of the *GC* gene variant rs7041 (*A* > *C*) and susceptibility to ASD, especially in individuals with both the *GC* gene rs7041 *C* allele and vitamin D insufficiency. Additionally, our study reveals an association between the *C* allele of the *GC* gene variant rs7041 (*A* > *C*), the *GC1s* isoform (rs7041*C*-rs4588*G*), and the *GC1s-GC1s* haplotype with both the risk and severity of ASD.

As a neurosteroid hormone, vitamin D is metabolized in the liver by vitamin D 25-hydroxylase (CYP2R1), resulting in the production of 25(OH)D (calcidiol), which is the main form of vitamin D circulating in the bloodstream. These metabolites remain inactive until they undergo conversion to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] by 25(OH)D 1α-hydroxylase (CYP27B1), primarily occurring in the proximal tubule of the kidney, after which they become biologically active[35]. At this stage, vitamin D can attach to the vitamin D receptor (VDR), present in various tissues and organs throughout the body such as enterocytes, osteoblasts, neurons, astrocytes, oligodendrocytes, and multiple brain regions[36,37].

Genetic variations in *CYP2R1* (rs10741657) and *CYP27B1* (rs4646536) may influence gene expression and enzyme activity, thereby affecting the level of 25(OH)D in serum[38,39]. However, in this study, we found no statistically significant associations between the genetic variants *CYP2R1* (rs10741657), *CYP27B1* (rs4646536), and the risk or severity of ASD. The lack of significant associations in our study may be due to genetic differences across populations or to environmental factors that were not accounted for. Further studies are needed to confirm the influence of genetic variants in *CYP2R1* and *CYP27B1* on the susceptibility to and severity of ASD.

For the *VDR* gene, located on chromosome 12q13 and consisting of nine exons and eight introns[40], the five single-nucleotide polymorphisms that are most frequently studied are *FokI* (rs2228570), *BsmI* (rs1544410), *ApaI* (rs7975232), *Cdx2* (rs11568820), and *TaqI* (rs731236). These SNPs have been shown to influence the structure and transcriptional and functional activity of VDR protein[26,27]. However, the effect of *VDR* gene polymorphisms on the susceptibility to and severity of ASD remains inconclusive[24,28–30]. The results of this study indicate that the polymorphism in the *VDR* gene rs7975232 (*ApaI*) was significantly associated with a reduced risk of ASD, consistent with a meta-analysis conducted in 2023, which reported that the rs7975232 polymorphism is one of the genetic factors associated with a reduced risk of ASD[41].

The vitamin D binding protein (VDBP), originally recognized as a group-specific component (GC), emerges as the most polymorphic protein known, with variations in the VDBP alleles exerting significant impacts on its biological functions. The most crucial among these are the two functional polymorphisms: rs7041 (c.1296*A* > *C*), which encodes glutamic acid instead of aspartic acid at position 432 (p.Asp432Glu), and rs4588 (c.1307*G* > *T*),

which encodes lysine instead of threonine at position 436 (p.Thr436Lys), both located in exon 11 of the VDBP gene[42]. These polymorphisms lead to the formation of three major GC isoforms : (1) *GC1s* (rs7041*C*-rs4588*G*), which encodes 432Glu/436Thr; (2) *GC1f* (rs7041*A* rs4588*G*), which encodes 432Asp/436Thr; and (3) *GC2* (rs7041*A*-rs4588*T*), which encodes 432Asp/436Lys. These three isoforms form six different haplotypes: *GC1f-GC1f*, *GC1f-GC1s*, *GC1s-GC1s*, *GC1f-GC2*, *GC1s-GC2*, and *GC2-GC2*. Our findings indicate a significant association between the susceptibility to ASD and its clinical severity and the rs7041 (*A* > *C*) polymorphism of the *GC* gene. Particularly noteworthy is the increased risk for ASD in individuals carrying the *GC* gene polymorphism rs7041 (*A* > *C*) along with vitamin D insufficiency. Additionally, our results demonstrate that the *GC1s* isoform and *GC1s-GC1s* haplotype are significantly more prevalent in children with ASD. Importantly, our study revealed that this specific isoform and haplotype are also significantly associated with the clinical severity of ASD.

The vitamin D binding protein facilitates the transportation of approximately 85% of the circulating vitamin D metabolites, while albumin binds around 15% of these metabolites with lower affinity. About 0.4% of total $1,25(OH)_2D$ and 0.03% of the total 25(OH)D are free in serum[21]. The term "total vitamin D" encompasses the sum of the free, albumin-bound, and VDBP-bound fractions of vitamin D. Bioavailable vitamin D refers to all circulating vitamin D that is not bound to VDBP, which includes both the free form and the form bound to albumin[43,44]. However, the extent to which the albumin fraction is genuinely bioavailable remains unclear[45]. According to the free hormone hypothesis, only the unbound fraction of hormones, which circulates in the bloodstream without being bound to carrier proteins, is able to enter cells and exert biological effects[44]. Vitamin D binding protein alleles exhibit variations in their affinity for 25(OH)D.

Bikle et al. observed the lowest free percentage of 25(OH)D with the *GC1s/GC1s* haplotype and the highest with the *GC1f/ GC1f* haplotype, suggesting a higher affinity of the *GC1s* allele for 25(OH)D compared to the Gc1f allele, with the *GC2* allele in between[21]. Similarly, Madden et al. found that VDBP levels are strongly influenced genetically, with carriers of the *GC1s* isoform having the highest VDBP levels and those carrying *GC1f* having the lowest VDBP levels[46]. This is consistent with the clinical trial study by Ganz et al., which reported that individuals with the *C* allele of the *GC* gene polymorphism rs7041 (*A* > *C*) exhibited significantly higher levels of VDBP and lower levels of free 25(OH)D[47]. Although the exact mechanisms linking vitamin D and autism remain uncertain, vitamin D is recognized as a neurosteroid that plays an active role in brain development. It influences cellular proliferation, differentiation, calcium signaling, and exhibits neurotrophic and neuroprotective actions. Additionally, it appears to affect neurotransmission and synaptic plasticity[48]. Furthermore, low vitamin D status is identified as an environmental risk factor for ASD[49]. Considering previous evidence in conjunction with our study findings, it may be hypothesized that individuals carrying the *GC* gene polymorphism rs7041 (*A* > *C*) or the *GC1s* isoform may have elevated VDBP levels, resulting in lower levels of the free fraction of 25(OH)D, impacting biological functions and diminishing the bioavailability of vitamin D, which could lead to an increased risk for ASD, as well as the increased clinical severity of ASD.

It is notable that VDBP may contribute to the pathogenesis of ASD through more than one mechanism. Recent findings indicate that the main effect of VDBP is to modulate inflammation, with only 2% of VDBP functioning as a vitamin binder[50]. VDBP is detectable in serum and cerebrospinal fluid[51], and it serves as a precursor of GcMAF, a protein that drives macrophage activation and transitions them into a proinflammatory phenotype. The transformation of VDBP into GcMAF is promoted by B and T lymphocytes and is mediated by a cascade of carbohydrate processing reactions[52]. Furthermore, VDBP can substantially augment the chemotactic activity of neutrophil chemoattractants both in vitro and in vivo[53]. Many studies have highlighted the association between ASD and neuroinflammation[9,10], an association further supported by growing clinical and experimental evidence linking disrupted immune and inflammatory responses to ASD pathogenesis[54]. Furthermore, Kern et al. identified chronic or excessive neuroinflammation in individuals with ASD, which may contribute to the behavioral characteristics of the disorder[55]. Lucchina et al. suggested that chronic inflammation, both peripherally and in the brain, could potentially lead to cognitive dysfunction[54]. Therefore, it might be concluded that individuals carrying the *GC* gene polymorphism rs7041 (*A* > *C*) or the *GC1s* isoform may have elevated VDBP levels[46,47], potentially leading to increased inflammation and an elevated risk of ASD and its clinical severity.

To date, research examining the influence of the *GC* gene on the risk and severity of ASD has been limited. Schmidt et al. reported that specific *GC* gene polymorphisms, particularly the rs4588 (*C* > *A*) *AA*-genotype/*A*-allele, were linked to ASD within the U.S. population[24]. In 2022, a study conducted in Italy found a significant rise in the prevalence of the *GC1f* isoform among children with ASD compared to healthy controls. The presence of *GC1f* and *GC1f-GC1f* was connected to more severe clinical manifestations of ASD[25]. The inconsistency among previous studies and our findings could be due to variations in the impact of polymorphisms on VDBP function across different ethnicities. Our study is the first to indicate the impact of the *GC* gene polymorphism and GC isoform on ASD susceptibility and its clinical severity within the Thai population.

The primary findings of this study indicate that the *VDR* gene ApaI polymorphism (rs7975232) is associated with a reduced risk of ASD. Conversely, individuals carrying the *GC* gene polymorphism rs7041 (A > C) and the GC1s isoform may have a heightened susceptibility to ASD and more severe clinical manifestations, particularly in the context of vitamin D insufficiency. Insights into genetic factors influencing the vitamin D pathway may facilitate personalized approaches for assessing the risk and prognosis of ASD. The *VDR* (ApaI) rs7975232 and *GC* (rs7041 A > C) polymorphisms could serve as predictive markers for ASD susceptibility and symptom severity. Furthermore, sufficient vitamin D supplementation or sun exposure may help reduce ASD risk in the general population and mitigate symptom severity in children with ASD.

However, our study has some limitations. Firstly, we measured total 25(OH)D using a chemiluminescent immunoassay and did not assess the VDBP levels. Despite this, mass spectrometry is increasingly recognized as the gold standard for measuring vitamin D metabolites[56,57] and is under development for quantifying VDBP and

its various isoforms[58,59]. Future studies should employ mass spectrometry to validate the association between the vitamin D levels and VDBP levels and the susceptibility to ASD, as well as its severity. Secondly, since Bioavailable 25(OH)D (comprising the free and albumin-bound fractions) has been suggested as a more accurate indicator of vitamin D activity[60,61], further investigation should include measurements of 'free' 25OHD and albumin levels. Thirdly, this study did not collect data on socioeconomic backgrounds or sun exposure, which might affect the susceptibility to ASD and vitamin D levels. Further studies need to include these variables. Fourthly, given that ASD is a complex disorder influenced by various genetic and environmental factors, including epigenetics, other factors beyond vitamin D may also contribute to susceptibility to ASD and its severity. Lastly, after applying false discovery rate (FDR) adjustment with the Benjamini-Hochberg procedure, the previously significant associations between the *GC* gene rs7041 (A > C) variant and ASD susceptibility and severity were no longer observed. The limited sample size of this study may have reduced its statistical power to detect associations between genetic polymorphisms and ASD susceptibility and severity. Therefore, replicating these findings in a larger sample or through multi-center collaborations is crucial to validate the associations between vitamin D gene polymorphisms and ASD susceptibility and severity.

Conclusion

In summary, this study is the first to demonstrate that the *VDR* gene (*ApaI*) rs7975232 is linked to a reduced risk of ASD. Furthermore, individuals with the *GC* gene polymorphism rs7041 (*A* > *C*) and the *GC1s* isoform may have increased susceptibility to ASD and greater clinical severity, particularly when combined with vitamin D insufficiency. Due to the limitations of a small sample size, further research involving larger, independent cohorts is necessary to validate these findings.

Methods

Participants

This case-control study involved 276 Thai children aged 2–18 years, with 169 children in the ASD group and 107 healthy children in the control group. The children with ASD were diagnosed according to the DSM-5 criteria by developmental pediatricians and experienced psychologists. The control group consisted of age- and gender-matched healthy Thai children, recruited from the same hospital, who were confirmed by the same developmental pediatricians to have no neurodevelopmental disorders. All participants were recruited from King Chulalongkorn Memorial Hospital between February 2022 and September 2023. The severity of symptoms was assessed using the Childhood Autism Rating Scale, 2nd Edition (CARS-2). CARS-2 comprises two parts: the CARS-2 Standard Version Rating Booklet (CARS2-ST), equivalent to the original CARS, and the CARS-2 High Functioning Version Rating Booklet (CARS2-HF), designed for individuals aged 6 years and older with an intelligence quotient above 80. The assessment involves 15 questions, each question is scored from 1 (normal at the corresponding age) to 4 (severely abnormal at the corresponding age). For CARS2-ST, a total score of 15-29.5 (15-27.5 for ages 13+) indicates minimal-to-no symptoms, 30–36.5 (28-34.5 for ages 13+) indicates mild to moderate autism, and a total score of ≥ 37 (35 and higher for ages 13+) indicates severe autism. For CARS2-HF, a total score of ≤ 27.5 indicates non-autism, a score between 28 and 33.5 indicates moderate autism, and a score of ≥ 34 indicates severe autism[32]. Subjects with a genetic syndrome, comorbid psychiatric disorders, childhood disintegrative disorder (CDD), congenital hypothyroidism, or a history of maternal infection were excluded from this study due to the potential impact of these conditions on neurodevelopment. Such disorders can significantly influence cognitive and behavioral outcomes, potentially mimicking ASD symptoms. Participants who had taken vitamin D supplements or medications affecting vitamin D levels within the past six months were excluded from total 25(OH)D level measurements to prevent altered vitamin D readings. Additionally, any missing data was excluded from the analysis to ensure the integrity and accuracy of the results. This study received approval from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (Approval Number 550/63) and was conducted in accordance with the Declaration of Helsinki. The authors confirm that all research was conducted in accordance with relevant guidelines and regulations. The study protocol was clearly explained to all patients, and informed consent was obtained from all participants, or from their parents or legal guardians for those under 17 years old, before their inclusion in the study. Blood samples were collected by trained personnel experienced in pediatric blood collection using sterile techniques. An EDTA tube was used for genotyping analysis, while a serum tube was used to measure vitamin D levels. The serum was stored at -80 °C to preserve vitamin D stability until analysis.

Genotyping methods

Peripheral blood mononuclear cells (PBMCs) were extracted from 6 mL of whole blood collected in an EDTA tube. DNA was isolated from PBMCs using GENEzol™ LS Reagent (Geneaid Biotech, New Taipei, Taiwan). The concentration and purity of the DNA were measured using a NanoDrop™ One spectrophotometer, assessing the 280/260 and 280/230 absorbance ratios. The sample was stored at -80 °C until analysis. For all SNPs genotypes (including the positive and negative controls), the *CYP2R1* gene (rs10741657), *CYP27B1* gene (rs4646536), *GC* gene (rs4588, rs7041), and *VDR* gene (rs731236, rs11568820, rs7975232, rs1544410, and rs2228570)), were identified using TaqMan® allelic discrimination methods real-time PCR assays (Applied Biosystems, Foster City, CA).

Evaluation of the serum vitamin D level

Vitamin D levels in the serum were measured using a chemiluminescent immunoassay (LIAISON® 25 OH Vitamin D TOTAL Assay) at the center for Medical Diagnostic Laboratories Faculty of Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The assessment of vitamin D levels in the blood followed

the Endocrine Society guidelines[62]. According to these criteria, sufficient vitamin D is defined as a 25(OH)D level greater than or equal to 30 ng/mL.

Statistical analysis

Genetic polymorphisms were assessed for concordance with Hardy–Weinberg equilibrium (HWE). Descriptive statistics were employed to characterize the clinical features of the subjects. To compare age and vitamin D levels between cases and controls, an unpaired t-test was performed (Table 1). Chi-square statistics were used to analyze the associations of genetic polymorphisms in vitamin D pathway genes with the risk and severity of ASD (Tables 2, 3, and 4; Supplementary Tables 1, 2, and 3). Chi-square statistics were also used to examine the association between GC gene isoforms, including their haplotypes, and the risk and severity of ASD (Tables 5 and 6; Supplementary Table 4). Additionally, multiple logistic regression analysis (forward stepwise method) was conducted to assess the predictive risk factors for ASD susceptibility and severity (Tables 7 and 8). All statistical analyses were conducted using IBM SPSS Statistics (Statistical Package for the Social Sciences, version 28.0.0, IBM Corp, Armonk, NY, USA; URL: https://www.ibm.com/products/spss-statistics). p values less than 0.05 were considered statistically significant. The p value was corrected for multiple comparisons using the Benjamini-Hochberg (BH) method to control the false discovery rate (FDR) (Supplementary Tables 1, 2 and 3).

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

C.Sa. performed all the experiments, collected samples and clinical data, analyzed the data, and drafted the manuscript. C.Sa., T.S., and N.V. contributed to funding acquisition. T.S. contributed to conceptualization, methodology, and supervision. W.C., P.T., and M.S. diagnosed and recruited the subjects. S.S. contributed to methodology and supervision. W.Y., C.P., Th.S., M.L.V.E., N.A., and S.T. collected samples and clinical data. R.S., P.S., and C.A. contributed to methodology. C.Su. and N.V. contributed to project administration, conceptualization, methodology, supervision, validation, and writing - review & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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