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Association of vitamin D-related gene polymorphisms with manifestation of vitamin D deficiency in children

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Abstract. The prevalence of vitamin D deficiency, presenting as hypocalcemic seizures or rickets in children, is increasing worldwide due to insufficient vitamin D intake and lack of exposure to sunshine. However, considering that relatively few children with low 25-hydroxyvitamin D [25(OH)D] levels manifest symptoms, it is possible that genetic factors may predispose individuals to vitamin D deficiency. Recent twin studies have reported that the level of serum of 25(OH)D is influenced by genetic factors. In addition, genome-wide association studies and candidate gene studies have revealed that several vitamin D-related genes, including *VDR, GC, NADSYN1*, *CYP2R1, CYP24A1*, *CYP27B1*, and *C10orf88* contribute to variations in serum 25(OH)D levels. To investigate whether genetic predisposition contributes to vitamin D deficiency, we analyzed polymorphisms in vitamin D-related genes in 30 Japanese patients with vitamin D deficiency presenting at less than 4 years of age, along with 66 controls. A χ^2 test showed that the genotype frequencies of *Bsm*I polymorphism in *VDR* and rs10898191 in *NADSYN1* were significantly different between the two groups. The allele frequencies of *Bsm*I, *Apa*I, *Taq*I in *VDR*, rs10898191 in *NADSYN1*, and rs705117 in *GC* were also significantly different. In particular, the frequency of the BAtS haplotype in *VDR* was significantly increased in the patient group relative to controls ($p = 0.0014$; odds ratio, 5.61; 95% confidence interval 1.92 - 16.40). Although this is a small study, our findings suggest that *VDR, NADSYN1*, and *GC* polymorphisms may be linked to the manifestation of vitamin D deficiency in Japanese children.

Key words: Vitamin D deficiency, Polymorphism, *VDR* gene, *GC* gene, *NADSYN1* gene

THE PREVALENCE of vitamin D deficiency, presenting as hypocalcemic seizures or rickets in children, is increasing worldwide due to insufficient intake of vitamin D, particularly by breast-feeding, and lack of exposure to sunshine. Vitamin D deficiency is most common in dark-skinned races, but it is also common in yellow-skinned races, including the Japanese [1-3].

Although a large number of breast-fed infants have low serum 25-hydroxyvitamin D [25(OH)D] levels, only a few of them manifest overt symptoms of vitamin D deficiency. Moreover, obvious environmental factors such as lack of exposure to sunshine or inadequate vitamin D intake are not associated with vitamin D deficiency in some patients. Therefore, we speculated that genetic predisposition may influence the manifestation of vitamin D deficiency.

Recent twin studies have reported that the level of serum of 25(OH)D is influenced by genetic factors [4-6]. The largest study reported that 70% of the variation in serum 25(OH)D levels during the winter is due to genetic factors. To date, many studies have reported the association of circulating 25(OH)D levels and polymorphisms in the genes encoding vitamin D-binding protein (*GC*), vitamin D receptor (*VDR*), vitamin D 1α-hydroxylase (*CYP27B1*), 24-hydroxylase (*CYP24A1*), and 25-hydroxylase (*CYP2R1*) by candidate gene analysis [7-10]. Moreover, recent genome-wide association studies (GWAS) have shown that several vitamin D-related genes, including NAD synthetase (*NADSYN1*, linked to 7-dehydrocholesterol reductase [*DHCR7*]), *GC*, *CYP2R1*, *CYP24A1*, and *C10orf88*, contribute to variations in serum 25(OH)D levels [11, 12], which association was replicated by others [13, 14]. However, few studies have investi-

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gated an association between gene polymorphisms and manifestation of vitamin D deficiency.

In this study, we analyzed previously reported vitamin D-related polymorphisms in Japanese children manifesting vitamin D deficiency.

Subjects and Methods

Subjects

Thirty Japanese patients manifesting vitamin D deficiency were included in the study. Selection criteria for patient participation in the study were as follows: vitamin D deficiency presenting at less than 4 years of age, vitamin D deficiency presenting with either overt rickets or hypocalcemic seizures, taking no medication that interferes with vitamin D metabolism, and having no history of rickets of prematurity. Clinical diagnosis of vitamin D deficiency was made by assessing hypocalcemia and/or hypophosphatemia, high serum alkaline phosphatase (ALP) levels, typical findings of rickets on bone roentgenogram, low circulating 25(OH)D levels, and elevated serum parathyroid hormone (PTH) levels. The control group consisted of 66 healthy Japanese volunteers with no history of rickets or hypocalcemic seizures.

The study protocol was approved by the ethical committees of the University of Tokyo and Yamagata University and was performed according to the Declaration of Helsinki. Written informed consent for DNA analyses was obtained from the parents of patients and healthy individuals.

Gene analysis

We selected polymorphisms that have been reported to be associated with serum 25(OH)D levels in either GWAS or candidate gene studies [8, 9, 11, 12, 15-20]. Well-known *VDR* polymorphisms reported to be associated with bone mineral density and several vitamin D-related diseases were also included [21-23]. Genomic DNA was isolated from peripheral white blood cells, and PCR-restriction fragment length polymorphism was performed for *VDR Bsm*I, *Apa*I, and *Taq*I (rs1544410, rs7975232, and rs731236, respectively) as previously reported [24]. *VDR* polyA polymorphism (rs17878969) was analyzed by GeneScan as previously reported [23], and based on the number of consecutive adenines (As) within the repeat sequence, polymorphism was classified as "L" (long), with more than 17 As, or "S" (short), with 17 or less As. PCR-direct sequencing was performed for other polymorphisms (*VDR Fok*I: rs10735810, *CYP27B1*: rs10877012; *CYP2R1*: rs10741657, rs2060793; *CYP24A1*: rs6013897; *GC*: rs4588, rs7041, rs2282679, rs1155563; *NADSYN1*: rs12785878, rs3829251; *C10orf88*: rs6599638), and were analyzed along with the neighboring single nucleotide polymorphisms (SNPs) (*GC*: rs705117; *NADSYN1*: rs10898191). The sequences of PCR primers and the PCR conditions will be provided on request.

Statistical analysis

Values are expressed as mean ± SD. The goodness of fit to the Hardy–Weinberg equilibrium was determined by comparing the calculated expected frequency of each genotype with the observed value using a χ^2 test. Comparisons of genotype frequency and allele frequency between patients and control subjects were performed using a χ^2 test for 2 × 2 or 2 × 3 tables, with Yates' correction if necessary. Genotype and the clinical data were compared using a Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

SNP analysis

The characteristics of the patient group were summarized in Table 1. Seven patients had no risk factors other than breast-feeding. None of the patients had

Table 1 Characteristics of patients

		Total (n=30)
Age at onset (years)*		1.48 ± 0.76
Male : female		26 : 4
Feeding in infancy	breast	25
	mixed	1
	unknown	4
Food restrictions/unbalanced diet	yes	18
	no	12
Low sunlight exposure	yes	5
	no	25
Serum 25(OH)D (ng/mL)**		median 8 (<5 to 22.9)
Serum ALP (IU/L)*		2,394 ± 699
Serum Ca (mg/dL)*		8.13 ± 1.61
Serum iP (mg/dL)*		3.84 ± 1.14
Serum intact PTH (pg/mL)*		272 ± 165

25(OH)D, 25-hydroxyvitamin D; ALP, alkaline phosphatase; Ca, calcium; iP, inorganic phosphate; *Mean ± SD **Serum 25(OH)D assay method was Diasorin radioimmunoassay, except for one with Diasorin Liaison Total Chemiluminescence immunoassay (7.82 ng/ml), and three with competitive protein-binding assay (11.8, 20.7, 22.9 ng/mL).

a mutation in the *CYP2R1* coding region (enzyme for vitamin D 25-hydroxylation) [25].

The distribution of the analyzed SNPs in the control group was in accordance with the assumption of Hardy-Weinberg equilibrium. We compared genotype and allele frequencies between patients and controls. Genotype frequencies of *Bsm*I polymorphism in *VDR* and rs10898191 in *NADSYN1* were significantly different between the patient and control groups (Table 2). The allele frequencies of the B allele of *Bsm*I, A allele of *Apa*I, and t allele of *Taq*I, all minor alleles of *VDR*, were significantly increased in the patient group ($p < 0.05$; Table 3). For other genes, the minor alleles of rs10898191 in *NADSYN1* and rs705117 in *GC* were significantly increased in the patient group ($p < 0.05$).

Haplotype analysis

*Bsm*I, *Apa*I, *Taq*I, and polyA polymorphisms in *VDR* lie near or within the last exon and are in linkage disequilibrium [26]. Because all alleles with increased frequency in the patients were minor alleles, we analyzed the frequency of the minor BAtS haplotype between the two groups and discovered a significantly increased frequency in the patient group relative to the control group ($p = 0.0014$; odds ratio, 5.61; 95% confidence interval 1.92 - 16.40; Table 4).

Association of genotype and clinical features

We compared age at onset, presence of risk factors other than breast-feeding, serum calcium levels, serum 25(OH)D levels, and serum 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] levels between patients with or without the BAtS haplotype or the high-risk alleles of rs10898191 and rs705117. However, we found no significant differences in any of these parameters (data not shown).

Discussion

We observed that polymorphisms in *VDR*, *GC*, and *NADSYN1* were associated with vitamin D deficiency. Moreover, patients harboring the minor BAtS haplotype had a 5.6-fold increased risk of vitamin D deficiency. The minor allele frequency of each *VDR* polymorphism in the control group was comparable to that in other Japanese controls, indicating no bias in the selection of the control group [27-29]. rs10898191 in *NADSYN1* is 42 bp away from rs3829251, which has been reported to be associated with reduced 25(OH)D levels in a GWAS [11]. Moreover, the association of rs3829251 with reduced 25(OH)D levels was also found in a Chinese cohort [14]. rs705117 in *GC* is 268 bp away from rs2282679, which is associated with reduced 25(OH)D levels in both Caucasians and Chinese [11, 12, 14]. We believe that polymorphisms in these genes are also associated with low 25(OH)D levels in the Japanese population.

The influence of environmental factors such as vitamin D intake or ultraviolet radiation levels on other diseases have been reported to differ with *VDR* genotype [23, 30]. Individuals with *VDR* minor genotypes are more sensitive to vitamin D administration and exhibit low calcium absorption [31, 32]. Moreover, *VDR* minor alleles have been reported to alter *VDR* function or expression [33, 34]. Otherwise, VDR-mediated regulation of 24-hydroxylase may be modified by altered VDR function, thereby influencing the degradation of 25(OH)D and $1,25(OH)_2D$. Taken our results together, we believe that children with *VDR* minor haplotype may be more influenced by vitamin D insufficiency and more likely to manifest overt rickets or hypocalcemia. This genetic predisposition may explain why only some of the thousands of children with low 25(OH)D levels manifest symptoms, while the majority are symptom free.

So far, many reports have shown an association between *VDR* polymorphisms and bone mineral density or osteoporosis [22, 35]. In particular, *Bsm*I, *Apa*I, *Taq*I, and polyA polymorphisms around the last exon are in linkage disequilibrium and have been extensively investigated. The two most relevant findings among several reports and a meta-analysis are that 1) the B allele of *Bsm*I is related to reduced bone mineral density and 2) Bat and BAt haplotypes are significantly associated with osteoporosis [36, 37]. The risk associated with a minor haplotype in reducing positive action of vitamin D is consistent with the results of this study.

Numerous reports indicate an association between *VDR* polymorphisms and diseases such as cancer and autoimmune diseases [22]. In particular, minor alleles around the last exon of *VDR* have been reported to be associated with type 1 diabetes, multiple sclerosis, and prostate cancer in Japanese patients [27-29, 38]. Vitamin D insufficiency is a risk factor for all these diseases [39]. Considering our results together, we believe that individuals with these minor alleles are at increased risk of the diseases because they are more influenced by vitamin D insufficiency. However,

Table 2 Genotype of single nucleotide polymorphisms analyzed in patients with vitamin D deficiency (n = 30) and controls (n = 66)

Gene	SNP	Group	Genotype			*p*-value
VDR	rs10735810		cc (FF)	ct (Ff)	tt (ff)	
	(*Fok*I)	Patient	15	12	3	0.889
		Control	28	28	10	
	rs1544410		gg (bb)	ag (bB)	aa (BB)	
	(*Bsm*I)	Patient	17	13	0	0.027*
		Control	54	10	2	
	rs7975232		gg (aa)	tg (aA)	tt (AA)	
	(*Apa*I)	Patient	6	19	5	0.052
		Control	32	25	9	
	rs731236		tt (TT)	tc (Tt)	cc (tt)	
	(*Taq*I)	Patient	17	13	0	0.063
		Control	53	12	1	
	rs17878969		LL	LS	SS	
	(polyA)	Patient	18	12	0	0.083
		Control	54	11	1	
CYP27B1	rs10877012		aa	ac	cc	
		Patient	17	11	2	0.892
		Control	41	19	6	
CYP2R1	rs10741657		aa	ag	gg	
		Patient	8	11	11	0.889
		Control	17	29	20	
	rs2060793		aa	ag	gg	
		Patient	5	13	12	0.980
		Control	9	32	25	
CYP24A1	rs6013897		tt	ta	aa	
		Patient	23	7	0	0.415
		Control	59	7	0	
GC	rs4588		cc	ca	aa	
		Patient	19	10	1	0.993
		Control	41	21	4	
	rs7041		tt	gt	gg	
		Patient	17	12	1	0.983
		Control	34	28	4	
	rs2282679		aa	ac	cc	
		Patient	15	12	3	0.814
		Control	40	22	4	
	rs705117		gg	ag	aa	
		Patient	7	12	11	0.075
		Control	24	33	9	
	rs1155563		tt	ct	cc	
		Patient	10	17	3	0.925
		Control	19	37	10	
NADSYN1	rs12785878		gg	gt	tt	
		Patient	13	13	4	0.982
		Control	30	28	8	
	rs3829251		gg	ag	aa	
		Patient	7	18	5	0.420
		Control	26	30	10	
	rs10898191		gg	ag	aa	
		Patient	4	20	6	0.043*
		Control	28	29	9	
c10orf88	rs659938		gg	ag	aa	
		Patient	7	8	15	0.475
		Control	10	28	28	

*$p < 0.05$

Table 3 Allele frequencies of the single nucleotide polymorphisms analyzed in patients with vitamin D deficiency (n = 30) and controls (n = 66)

Gene	SNP	Group	Allelic frequency		*p*-value	OR (95% CI)
VDR	rs10735810		c (F)	t (f)		
	(*Fok*I)	Patient	0.70	0.30	0.390	0.75 (0.39-1.45)
		Control	0.64	0.36		
	rs1544410		g (b)	a (B)		
	(*Bsm*I)	Patient	0.78	0.22	0.041*	2.33 (1.02-5.33)
		Control	0.89	0.11		
	rs7975232		g (a)	t (A)		
	(*Apa*I)	Patient	0.52	0.48	0.037*	1.94 (1.04-3.61)
		Control	0.67	0.33		
	rs731236		t (T)	c (t)		
	(*Taq*I)	Patient	0.78	0.22	0.041*	2.33 (1.02-5.33)
		Control	0.89	0.11		
	rs17878969		L	S		
	(polyA)	Patient	0.80	0.20	0.053	2.29 (0.97-5.37)
		Control	0.90	0.10		
CYP27B1	rs10877012		a	c		
		Patient	0.75	0.25	0.819	1.09 (0.53-2.21)
		Control	0.77	0.23		
CYP2R1	rs12794714		c	t		
		Patient	0.55	0.45	0.738	1.11 (0.60-2.05)
		Control	0.58	0.42		
	rs10741657		g	a		
		Patient	0.55	0.45	0.725	0.90 (0.49-1.65)
		Control	0.52	0.48		
	rs2060793		g	a		
		Patient	0.62	0.38	0.952	1.02 (0.54-1.91)
		Control	0.62	0.38		
CYP24A1	rs6013897		t	a		
		Patient	0.88	0.12	0.203	2.36 (0.79-7.01)
		Control	0.95	0.05		
GC	rs4588		c	a		
		Patient	0.80	0.20	0.758	0.89 (0.42-1.89)
		Control	0.78	0.22		
	rs7041		t	g		
		Patient	0.77	0.23	0.564	0.81 (0.40-1.65)
		Control	0.73	0.27		
	rs2282679		a	c		
		Patient	0.70	0.30	0.280	1.46 (1.73-2.89)
		Control	0.77	0.23		
	rs2282680		g	a		
		Patient	0.77	0.23	0.833	1.08 (0.52-2.24)
		Control	0.78	0.22		
	rs705117		g	a		
		Patient	0.43	0.57	0.020*	2.07 (1.12-3.86)
		Control	0.61	0.39		
	rs1155563		t	c		
		Patient	0.62	0.38	0.527	0.82 (0.44-2.05)
		Control	0.57	0.43		
NADSYN1	rs12785878		g	t		
		Patient	0.65	0.35	0.821	1.08 (0.57-2.05)
		Control	0.67	0.33		
	rs3829251		g	a		
		Patient	0.53	0.47	0.250	1.44 (0.77-2.66)
		Control	0.62	0.38		
	rs10898191		g	a		
		Patient	0.47	0.53	0.021*	2.07 (1.11-3.84)
		Control	0.64	0.36		
	rs71473837		a	g		
		Patient	0.93	0.07	0.786	0.98 (0.29-3.30)
		Control	0.93	0.07		
c10orf88	rs659938		a	g		
		Patient	0.63	0.37	0.903	1.01 (0.54-1.91)
		Control	0.64	0.36		

*p <0.05, OR, odds ratio, 95% CI 95% confidence interval

Table 4 BAtS haplotype in *VDR* of patients with vitamin D deficiency (n = 30) and controls (n = 66)

Group	BAtS -	BAtS +	*p*-value	OR (95% CI)
Patient	18	12	0.0014*	5.61 (1.92-16.40)
Control	59	7		

*$p < 0.05$, OR, odds ratio; 95% CI, 95% confidence interval

larger studies are necessary to confirm the relationship between the risk of vitamin D deficiency in childhood and other diseases related to vitamin D insufficiency.

Some studies investigating the genetic factors involved in vitamin D-deficient rickets identified association with *VDR Apa*I and *Fok*I polymorphisms, whereas others found no association with *Bsm*I, *Apa*I, and *Taq*I polymorphisms [40-43]. Additionally, an almost significant degree of association has been reported between *Apa*I polymorphism and osteomalacia [44]. Although these associations are not identical to our data, probably because of differences in ethnicity, we believe that *VDR* is most likely to be one of the disease susceptibility genes for vitamin D deficiency.

One of the limitations of our study was the relatively small sample size. Except for the haplotype analysis, no disease associations with polymorphisms reached a level of significance after correction for multiple testing. Another limitation was the lack of a comprehensive genetic analysis, which needs a far larger sample cohort. Moreover, we did not identify any clinical differences between patients with and without risk-associated haplotypes. This may have been because our group included only young children, and further analyses including patients of different ages may be necessary to clarify this point.

Recently, vitamin D supplementation has been recommended for all infants, especially those who are breast-fed [45]. However, some children are genetically sensitive to vitamin D supplementation and may become hypercalcemic after this treatment [46]. Together with our finding of genetic predisposition for vitamin D deficiency, we believe that genetic factors should be taken into account in the future design of personalized supplementation. In addition, large studies on patients with overt symptoms of vitamin D deficiency are necessary.

In summary, although only a limited number of subjects were investigated, we found an association of *VDR*, *GC*, and *NADSYN1* polymorphisms with vitamin D deficiency. Considering that only some children with reduced serum 25(OH)D levels manifest overt symptoms of vitamin D deficiency, our findings suggest that polymorphisms of these genes may be linked to the manifestation of vitamin D deficiency in Japanese children.

Conflict of Interest

All authors have no conflicts of interest.

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