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The functional polymorphisms of *VDR*, *GC* and *CYP2R1* are involved in the pathogenesis of autoimmune thyroid diseases

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Summary

Vitamin D is a multi-functional immune regulator, and a low serum concentration of vitamin D promotes autoimmune inflammation. In this study, we evaluate the association between the prognosis of autoimmune thyroid disease (AITD) and the functional polymorphisms of genes that regulate vitamin D metabolism. For 139 Graves' disease (GD) patients, 116 Hashimoto's disease (HD) patients and 76 control subjects, we genotyped the following polymorphisms using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP): vitamin D receptor (VDR): rs731236, rs7975232, rs2228570 and rs1544410; group-specific component (GC): rs7041 and rs4588; and CYP2R1: rs10741657. The frequency of the TT genotype for the rs731236 polymorphism was higher in GD patients than in HD patients ($P = 0{\cdot}0147$). The frequency of the C allele for the rs7975232 polymorphism was higher in GD patients than in control subjects ($P = 0{\cdot}0349$). The proportion of GD patients whose anti-thyrotrophin receptor antibody (TRAb) level was >51% was higher in those with the CC genotype than in those with the CA+AA genotypes ($P = 0{\cdot}0065$). The frequency of the CC genotype for the rs2228570 polymorphism was higher in HD patients than in control subjects ($P = 0{\cdot}0174$) and GD patients ($P = 0{\cdot}0149$). The frequency of the Gc1Gc1 genotype for the *GC* polymorphism and the AG genotype for the *CYP2R1* polymorphism were lower in intractable GD than in GD in remission ($P = 0{\cdot}0093$ and $0{\cdot}0268$, respectively). In conclusion, genetic differences in the *VDR* gene may be involved in the development of AITD and the activity of GD, whereas the genetic differences in the *GC* and *CYP2R1* genes may be involved with the intractability of GD.

Keywords: autoimmune thyroid disease, intractability, polymorphism, severity, vitamin D

Introduction

Autoimmune thyroid diseases (AITDs), such as Hashimoto's disease (HD) and Graves' disease (GD), are archetypes of organ-specific autoimmune disease [1,2]. The severity of HD and intractability of GD vary among patients. Some patients develop hypothyroidism in early life, and some maintain a euthyroid state in old age despite the passage of time. Some patients with GD achieve remission through medical treatment; others do not. Although it is difficult to predict the disease severity and intractability of AITDs, we have reported previously that they are affected by the patient's genetic capability to express and produce immune regulatory factors [3–11].

Vitamin D is a multi-functional immune regulator that has been shown to inhibit the differentiation of T helper type 1 (Th1) and Th17 cells and to promote the generation of regulatory T (T_{reg}) cells [12,13]. The serum concentrations of $25(OH)_2D_3$ and 1·25-dihydroxy vitamin D_3 [$1{\cdot}25(OH)_2D_3$], which are metabolites of vitamin D [14], have been shown to be lower in patients with autoimmune diseases, including AITDs and multiple sclerosis (MS) [15–17]. These studies suggested that reduced immune regulation of vitamin D may induce autoimmune reactions.

Table 1. The function of *VDR* polymorphism genotyped in this study.

Polymorphism	The function of polymorphism	References
rs7975232 C/A (ApaI)	AA genotype > CC genotype [serum 25 $(OH)_2D_3$ concentration]	[19]
rs1544410 A/G (BsmI)	A allele > G allele (IFN-γ production in PBMC)	[20]
rs2228570 C/T (FokI)	TT genotype > CC genotype [serum 25 $(OH)_2D_3$ concentration]	[19]
rs731236 T/C (TaqI)	T allele > C allele (VDR expression)	[21]

IFN = interferon; PBMC = peripheral blood mononuclear cells.

Vitamin D receptor (VDR) is a nuclear receptor that binds specific DNA sequences and vitamin D response elements (VDRE), and controls the transcription of regulating genes associated with calcium metabolism and immune responses [18]. The major polymorphisms of the *VDR* gene are rs7975232 (ApaI), rs1544410 (BsmI), rs2228570 (FokI) and rs731236 (TaqI). The functions of these polymorphisms are summarized in Table 1[19–21]. For the rs7975232 polymorphism, the frequency of the C allele is higher in Egyptians with GD than in healthy controls [15], and lower in Japanese with GD than in healthy controls [22]. For the rs1544410 polymorphism, the A allele was reported to be a risk allele for type I diabetes (T1D) and GD [20,22]. For the rs2228570 polymorphism, the frequency of the C allele was higher in Chinese with GD than in healthy controls and in Japanese with HD than in healthy controls [23,24].

Genome-wide association studies (GWAS) have shown that group-specific components (*GC*) and *CYP2R1* are associated with circulating vitamin D levels [25,26]. The *GC* gene encodes vitamin D binding protein (DBP), a vitamin D transporter. There are two common polymorphisms (rs4588 and rs7041) in the *GC* gene that are associated with the binding affinity of GC to vitamin D [27]. There are three major isoforms of DBP, Gc1F, Gc1S and Gc2. A threonine substituted by lysine (rs4588) generates Gc2, and an aspartic acid substituted by glutamine (rs7041) generates Gc1S. The binding affinity of Gc1F to vitamin D was four times higher than that of Gc2 and two times higher than that of Gc1S [27]. In addition, the serum and plasma vitamin D concentration in Gc2 allele carriers was lower than that in Gc1 allele (Gc1F + Gc1S) carriers [28,29]. Therefore, rs4588 and rs7041 are functional polymorphisms because they allow the generation of Gc1 and Gc2 isoforms. Conversely, the functional polymorphisms in the *CYP2R1* gene, rs12794714 and rs10741657, are in linkage disequilibrium [30,31]. The vitamin D serum concentration in individuals with the AA genotype of rs10741657 was higher than that in G carriers [31], while individuals with the GG genotype had a higher vitamin D serum concentration than A carriers [30]. The functions of this polymorphism have not been clarified.

In this study, we genotyped these functional polymorphisms to clarify the association of genes that regulate vitamin D for the development and prognosis of AITD.

Materials and methods

Subjects

We carefully selected 54 patients with severe HD and 42 patients with mild HD and 20 patients who could not be categorized to severe HD or mild HD groups at the time of analysis from 116 HD patients who were positive for antithyroid microsomal antibody (McAb) and/or antithyroglobulin antibody (TgAb). Patients with HD who developed moderate to severe hypothyroidism before 50 years of age and who were treated daily with thyroxine were defined as patients with severe HD, and untreated, euthyroid patients with HD who were more than 50 years of age were defined as patients with mild HD. All patients with mild HD had a palpable diffuse goitre.

We also carefully selected 61 patients with intractable GD, 40 patients with GD in remission and 38 patients who could not be categorized to intractable GD or GD in remission groups at the time of analysis from 139 GD patients who had a clinical history of thyrotoxicosis with elevated TRAb. Euthyroid patients with GD who had been treated with methimazole for at least 5 years and were still positive for TRAb were defined as patients with intractable GD, and patients with GD who had maintained a euthyroid state and were negative for TRAb for more than 2 years without medication were defined as patients with GD in remission. We genotyped 76 healthy volunteers (control subjects) who were euthyroid and negative for thyroid autoantibodies. Written informed consent was obtained from all patients and controls, and the study protocol was approved by the Ethics Committee of Osaka University. Clinical characteristics of subjects were shown in Table 2.

Genotyping of polymorphisms

The polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method was used for genotyping each polymorphism. The target sequences of each gene were amplified using PCR, and the resulting PCR products were digested by addition of restriction enzymes. The sequences of the forward and reverse primers, the PCR conditions and the restriction enzymes used are summarized in Table 3.

Table 2. Clinical characteristics of groups of patients with autoimmne thyroid disease at the time of sampling.

		Graves' disease (GD)		Hashimoto's disease (HD)	
		Past clinical history of thyrotoxicosis with elevated TRAb		Diffuse goitre and positive TgAb and/or McAb	
	Controls	Intractable	In remission	Severe	Mild
n (female/male)	76 (49/27)	61 (49/12)	40 (30/10)	54 (43/11)	42 (34/8)
Age of onset (years)	28·9 ± 11·0‡	33·2 ± 13·6	30·8 ± 11·1	37·2 ± 11·0	59·0 ± 10·2‡
(range)	(21–58)	(11~66)	(10~58)	(10~49)	(50~92)
Free T4 (ng/dl) (normal range: 0·9–1·7 ng/dl)	n.d.	1·25 ± 0·31	1·24 ± 0·16	1·36 ± 0·30	1·24 ± 0·23
Free T3 (pg/ml) (normal range: 2·3–4·3 pg/ml)	n.d.	2·76 ± 0·55	2·64 ± 0·34	2·59 ± 0·56	2·78 ± 0·42
TSH (μU/ml) (normal range: 0·5–5·0 μU/ml)	n.d.	1·60 ± 1·27	1·87 ± 1·26	1·83 ± 1·32	2·67 ± 1·78
TRAb (IU/l)	<2·0	7·64 ± 13·1	<2·0	<2·0	<2·0
(range)		(2·0~71)			
TgAb ($2^n \times 100$)*	Negative	2·89 ± 2·93	2·48 ± 0·64	7·00 ± 3·49	1·56 ± 2·40
McAb ($2^n \times 100$)*	Negative	4·73 ± 2·59	2·71 ± 0·96	5·41 ± 3·18	3·23 ± 2·77
Current treatment	None	MMI or PTU	None	L-thyroxine	None
Treatment time (years)	None	12·1 ± 7·61	3·21 ± 1·26‡	11·3 ± 8·52	None
Current dose of anti-thyroid drug (mg/day)†	None	19·1 ± 34·4	None	None	None
(range)		(2·5~200)			
Current dose of L-thyroxine (μg/day)	None	None	None	90·4 ± 37·7	None
(range)				(50~250)	

*When the titre of anti-thyroglobulin antibody (TgAb) or microsomal antibody (McAb) was 25600, it was expressed as 28 × 100, †Doses were expressed as the comparable dose of methimazole (MMI) [50 mg of propylthiouracil (PTU) was converted to 5 mg of MMI]. ‡Age at the time of sampling. Data are expressed as mean ± standard deviation. McAb = anti-thyroid microsomal antibody; n.d. = not determined; T3 = triiodothyronine; T4 = thyroxine; TRAb = anti-thyrotrophin receptor antibody; TSH = thyrotrophin.

Thyroid function and autoantibodies

The serum concentration of free T4 (FT4), free T3 (FT3) and thyrotrophin (TSH) were measured with electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics Ltd, Tokyo, Japan). The normal serum concentration ranges of FT4, FT3 and TSH are 0·9–1·7 ng/dl, 2·3–4·3 pg/ml and 0·5–5·0 μU/ml, respectively. TgAb and McAb were measured with a particle agglutination kit (Fujirebio Inc., Tokyo, Japan). A reciprocal titre of >1 : 100

Table 3. The primers, polymerase chain reaction (PCR) conditions and restricion enzymes used in this study.

		Primer pairs	PCR conditions	Restriction enzymes
VDR	rs7975232 (ApaI)	5′-CAGAGCATGGACAGGGAGCAA-3′ 5′-GCAACTCCTCATGGCTGAGGTCTC-3′	95°C for 5 min (95°C for 30 s, 70°C for 30 s, 72°C for 60 s) × 35cycles 72°C for 5 min	ApaI
	rs1544410 (BsmI)	5′-CAACCAAGACTACAAGTACCGCGTCAGTGA-3′ 5′-AACCAGCGGGAAGAGGTCAAGGG-3′	95°C for 3 min (95°C for 30 s, 60°C for 30 s, 72°C for 30 s) × 35cycles 72°C for 5 min	BsmI
	rs2228570 (FokI)	5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′	95°C for 5 min (95°C for 30 s, 61°C for 30 s, 72°C for 30 s) × 35cycles 72°C for 5 min	FokI
	rs731236 (TaqI)	5′-CAGAGCATGGACAGGGAGCAA-3′ 5′-GCAACTCCTCATGGCTGAGGTCTC-3′	95°C for 5 min (95°C for 30 s, 70°C for 30 s, 72°C for 60 s) × 35cycles 72°C for 5 min	TaqI
GC	rs7041	5′-AAATAATGAGCAAATGAAAGAAGAC-3′ 5′-CAATAACAGCAAAGAAATGAGTAGA-3′	95°C for 3 min (95°C for 30 s, 64°C for 30 s, 72°C for 30 s) × 30cycles 72°C for 7 min	HaeIII
	rs4588	5′-AAATAATGAGCAAATGAAAGAAGAC-3′ 5′-CAATAACAGCAAAGAAATGAGTAGA-3′	95°C for 3 min (95°C for 30 s, 64°C for 30 s, 72°C for 30 s) × 30cycles 72°C for 7 min	StyI
CYP2R1	rs10741657	5′-GGGAAGAGCAATGACATGGA-3′ 5′-GCCCTGGAAGACTCATTTTG-3′	95°C for 5 min (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) × 35cycles 72°C for 5 min	MnlI

Table 4. Genotype and allele frequencies of the polymorphisms genotyped in patients with Graves' disease (GD), Hashimoto's disease (HD) and in control subjects.

		Control	All patients with AITD		All patients with GD		All patients with HD	
VDR	TT	58 (77·3%)	196 (81·3%)		109 (87·2%)	0·0147†§	87 (75·0%)	
rs731236	TC	17 (22·7%)	43 (17·9%)	n.s.*	15 (12·0%)	n.s.*	28 (24·1%)	n.s.*
	CC	0 (0%)	2 (0·8%)		1 (0·8%)		1 (0·9%)	
	T allele	133 (88·7%)	435 (90·2%)	n.s.*	233 (93·2%)	n.s.*	202 (87·1%)	n.s.*
	C allele	17 (11·3%)	47 (9·8%)		17 (6·8%)		30 (12·9%)	
VDR	CC	31 (41·3%)	114 (49·1%)		63 (50·4%)		51 (47·7%)	
rs7975232	CA	32 (42·7%)	105 (45·3%)	0·0256*	56 (44·8%)	0·0280*	49 (45·8%)	n.s.*
	AA	12 (16·0%)	13 (5·6%)		6 (4·8%)		7 (6·5%)	
	C allele	94 (62·7%)	333 (71·8%)	0·037*	182 (72·8%)	0·0349*	151 (70·6%)	n.s.*
	A allele	56 (37·3%)	131 (28·2%)		68 (27·2%)		63 (29·4%)	
VDR	CC	25 (32·9%)	100 (41·8%)		46 (34·9%)		54 (50·5%)	0·0174*¶, 0·0149‡¶
rs2228570	CT	42 (55·3%)	113 (47·3%)	n.s.*	70 (53·0%)	n.s.*	43 (40·2%)	n.s.*
	TT	9 (11·8%)	26 (10·9%)		16 (12·1%)		10 (9·3%)	
	C allele	92 (59·2%)	313 (65·5%)	n.s.*	162 (61·4%)	n.s.*	151 (70·6%)	0·0458*, 0·0348‡
	T allele	60 (40·8%)	165 (34·5%)		102 (38·6%)		63 (29·4%)	
VDR	AA	3 (4·7%)	7 (3·4%)		3 (2·7%)		4 (4·1%)	
rs1544410	AG	11 (17·2%)	36 (17·3%)	n.s.*	15 (13·7%)	n.s.*	21 (21·4%)	n.s.*
	GG	50 (78·1%)	165 (79·3%)		92 (83·6%)		73 (74·5%)	
	A allele	17 (13·3%)	50 (12·0%)	n.s.*	21 (9·5%)	n.s.*	29 (14·8%)	n.s.*
	G allele	111 (86·7%)	366 (88·0%)		199 (90·5%)		167 (85·2%)	
GC	Gc1Gc1	37 (61·7%)	131 (54·6%)		64 (50·8%)		67 (58·8%)	
	Gc1Gc2	19 (31·7%)	97 (40·4%)	n.s.*	54 (42·9%)	n.s.*	43 (37·7%)	n.s.*
	Gc2Gc2	4 (6·6%)	12 (5·0%)		8 (6·3%)		4 (3·5%)	
	Gc1 allele	93 (77·5%)	359 (74·8%)	n.s.*	182 (72·2%)	n.s.*	177 (77·6%)	n.s.*
	Gc2 allele	27 (22·5%)	121 (25·2%)		70 (27·8%)		51 (22·4%)	
CYP2R1	GG	25 (40·3%)	97 (38·2%)		54 (38·9%)		43 (37·4%)	
rs10741657	GA	26 (41·9%)	111 (43·7%)	n.s.*	60 (43·2%)	n.s.*	51 (44·4%)	n.s.*
	AA	11 (17·8%)	46 (18·1%)		25 (17·9%)		21 (18·2%)	
	G allele	76 (61·3%)	305 (60·3%)	n.s.*	168 (60·4%)	n.s.*	137 (59·6%)	n.s.*
	A allele	48 (38·7%)	203 (39·7%)		110 (39·6%)		93 (40·4%)	

Analysed by χ^2 tests **versus* control; †*versus* HD; ‡*versus* GD; §TT *versus* TC + CC; ¶CC *versus* CT + TT; n.s. = not significant. AITD = autoimmune thyroid disease.

was considered positive. Serum TRAb concentration at the time of disease onset was measured by a radioreceptor assay with a commercial kit (Cosmic Corporation, Tokyo, Japan) as part of a routine study. Serum TRAb concentration during sampling was determined with ECLIA (third-generation) (Roche Diagnostics Ltd). The normal value of TRAb was less than 10% by the radioreceptor assay and 2·0 IU/l by ECLIA.

Statistical analysis

We used the χ^2 test and Fisher's exact test to evaluate the significance of the differences in the frequencies of genotypes and alleles among the different groups. Student's *t*-test was used to analyse differences in goitre size and serum TRAb levels. The differences in titres of TgAb and McAb were analysed using the Mann–Whitney *U*-test. The data were analysed with JMP10 software (SAS Institute, Inc., Tokyo, Japan). Probability values of less than 0·05 were considered significant.

Results

VDR polymorphisms

The frequency of the TT genotype for the rs731236 polymorphism was higher in GD patients than in HD patients ($P = 0·0147$). The C allele frequency for the rs7975232 polymorphism was higher in AITD patients, especially GD patients, than in control subjects ($P = 0·0375$ and 0·0349, respectively) (Table 4). The frequencies of the CC genotype and C allele for the rs2228570 polymorphism were higher in HD patients than in control subjects ($P = 0·0174$ and 0·0458, respectively) or GD patients ($P = 0·0149$ and 0·0348, respectively) (Table 4). We found no difference in the frequencies of the genotypes and alleles of the rs1544410 polymorphism between the control subjects and the patients in the HD and GD groups. The frequencies of the genotypes and alleles in these VDR polymorphisms did not differ between the patients with severe HD and those with mild HD or between the patients with intractable GD and those

Table 5. Genotype and allele frequencies of the polymorphisms genotyped in patients with Graves' disease (GD) and Hashimoto's disease (HD).

			GD				HD		
		Control	Intractable	In remission			Severe	Mild	
VDR	TT	58 (77·3%)	45 (84·9%)	32 (86·5%)			42 (79·3%)	30 (75·0%)	
rs731236	TC	17 (22·7%)	7 (13·2%)	5 (13·5%)	n.s.*		10 (18·9%)	10 (25·0%)	n.s.‡
	CC	0 (0%)	1 (1·9%)	0 (0%)			1 (1·8%)	0 (0%)	
	T allele	133 (88·7%)	97 (91·5%)	69 (93·2%)	n.s.*		94 (88·7%)	70 (87·5%)	n.s.‡
	C allele	17 (11·3%)	9 (8·5%)	5 (6·8%)			12 (11·3%)	10 (12·5%)	
VDR	CC	31 (41·3%)	25 (50·0%)	16 (41·0%)			22 (50·0%)	18 (42·9%)	
rs7975232	CA	32 (42·7%)	23 (46·0%)	19 (48·7%)	n.s.*		18 (40·9%)	22 (52·4%)	n.s.‡
	AA	12 (16·0%)	2 (4·0%)	4 (10·3%)			4 (9·1%)	2 (4·7%)	
	C allele	94 (62·7%)	73 (73·0%)	51 (65·4%)	n.s.*		62 (70·5%)	58 (69·0%)	n.s.‡
	A allele	56 (37·3%)	27 (27·0%)	27 (34·6%)			26 (29·5%)	26 (31·0%)	
VDR	CC	25 (32·9%)	18 (36·7%)	9 (22·5%)			20 (45·5%)	22 (55·0%)	
rs2228570	CT	42 (55·3%)	27 (55·1%)	27 (67·5%)	n.s.*		19 (43·2%)	16 (40·0%)	n.s.‡
	TT	9 (11·8%)	4 (8·2%)	4 (10·0%)			5 (11·3%)	2 (5·0%)	
	C allele	92 (59·2%)	63 (64·3%)	45 (56·3%)	n.s.*		59 (67·0%)	60 (75·0%)	n.s.‡
	T allele	60 (40·8%)	35 (35·7%)	35 (43·7%)			29 (33·0%)	20 (25·0%)	
VDR	AA	3 (4·7%)	0 (0%)	1 (2·8%)			0 (0%)	2 (5·4%)	
rs1544410	AG	11 (17·2%)	4 (9·5%)	6 (16·7%)	n.s.*		7 (17·1%)	9 (24·3%)	n.s.‡
	GG	50 (78·1%)	38 (90·5%)	29 (80·5%)			34 (82·9%)	26 (70·3%)	
	A allele	17 (13·3%)	4 (4·5%)	8 (11·1%)	n.s.*†		7 (8·5%)	13 (17·6%)	n.s.‡
	G allele	111 (86·7%)	80 (95·5%)	64 (88·9%)			75 (91·5%)	61 (82·4%)	
GC	Gc1Gc1	37 (61·7%)	21 (38·9%)	24 (66·7%)	0·0093*§		31 (62·0%)	25 (62·5%)	
	Gc1Gc2	19 (31·7%)	32 (59·3%)	10 (27·8%)	0·0109*		18 (36·0%)	15 (37·5%)	n.s.‡
	Gc2Gc2	4 (6·6%)	1 (1·8%)	2 (5·5%)			1 (2·0%)	0 (0%)	
	Gc1 allele	93 (77·5%)	74 (68·5%)	58 (80·6%)	n.s.*		80 (80·0%)	65 (81·3%)	n.s.‡
	Gc2 allele	27 (22·5%)	34 (31·5%)	14 (19·4%)			20 (20·0%)	15 (18·7%)	
CYP2R1	GG	25 (40·3%)	26 (42·6%)	12 (30·0%)			20 (37·0%)	11 (30·5%)	
rs10741657	GA	26 (41·9%)	20 (32·8%)	22 (55·0%)	n.s.*	0·0268*¶	23 (42·6%)	19 (52·8%)	n.s.‡
	AA	11 (17·8%)	15 (24·6%)	6 (15·0%)			11 (20·4%)	6 (16·7%)	
	G allele	76 (61·3%)	72 (59·0%)	46 (57·5%)	n.s.*		63 (58·3%)	41 (56·9%)	n.s.‡
	A allele	48 (38·7%)	50 (41·0%)	34 (42·5%)			45 (41·7%)	31 (43·1%)	

Analysed by χ^2 tests or †Fisher's exact test; *intractable GD *versus* GD in remission; ‡severe HD *versus* mild HD; §Gc1Gc1 *versus* Gc1Gc2 + Gc2Gc2; ¶GA *versus* GG + AA; n.s. = not significant.

with GD in remission (Table 5). The frequency of the CC genotype for the rs2228570 polymorphism tends to be higher in patients with severe HD than in control subjects, but not significant ($P = 0{\cdot}17$).

GC polymorphism

We categorized the Gc1 (Gc1F + Gc1S) and Gc2 alleles according to the results for the rs7041 and rs4588 polymorphisms. The frequency of the Gc1Gc1 genotype was lower in patients with intractable GD than in those with GD in remission ($P = 0{\cdot}0093$) (Table 5), although we found no difference in the genotype or allele frequencies of this polymorphism between the control subjects and the patients in the HD and GD groups. These frequencies did not differ between patients with severe HD and those with mild HD (Tables 4 and 5).

CYP2R1 polymorphism

The frequency of the AG genotype of the rs10741657 polymorphism was lower in patients with intractable GD than in those with GD in remission ($P = 0{\cdot}0268$) (Table 5). We found no differences in the frequencies of the genotypes or alleles of this polymorphism between the control subjects and the patients in the HD and GD groups. These frequencies did not differ between patients with severe HD and those with mild HD (Tables 4 and 5).

Clinical characteristics and genotype frequencies

For the rs7975232 polymorphism, the proportion of GD patients whose anti-TRAb level at the onset of disease was >51% was significantly higher for patients with the CC genotype (58·3%) than in those with the AA + CA

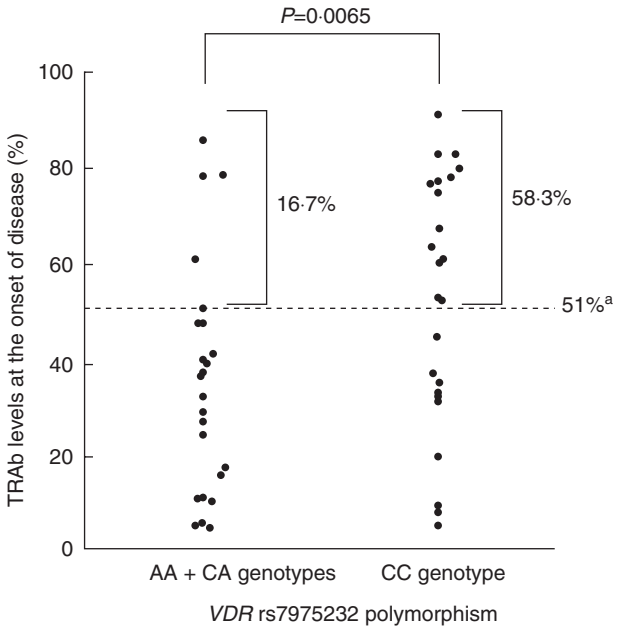

Fig. 1. Serum levels of thyrotrophin receptor antibody (TRAb) at the onset of disease in Graves' disease (GD) patients with different *VDR* rs7975232 genotypes. (a) We set the cut-off value for TRAb level at the onset of disease at 51%.

genotypes (16·7%) ($P = 0{\cdot}0065$) (Fig. 1). However, we found no association between TRAb level, anti-thyroid McAb titre, anti-TgAb titre or goitre size and any other polymorphisms.

Discussion

In this study, the frequencies of the CC genotype and C allele for the *VDR* rs2228570 polymorphism, which were correlated with a lower concentration of serum vitamin D, were higher in the HD group than in control subjects (Table 4). These data are consistent with that of a previous report [23]. Because the C allele was also associated with a higher production of interleukin (IL)-12, which induces Th1 differentiation [32] and thyroid destruction in HD patients, possibly through cytotoxic T lymphocytes and Th1 cells [33,34], the CC genotype may be associated with the induction of autoimmune thyroid destruction. Conversely, the frequency of the C allele for the *VDR* rs7975232 polymorphism was higher in AITD patients, especially GD patients (Table 3). In a previous study, however, the frequency of this allele was lower in GD patients [22]. We randomly selected a sample of subjects and genotyped this polymorphism again using the direct sequencing method, and we confirmed all our results. Therefore, we concluded that the frequency of the C allele may be higher in GD patients, and our results may have been inconsistent with those of the previous study because of differences in the GD patient populations of the two studies. In our study, interestingly, the TRAb level at the onset of disease was significantly higher in GD patients with the CC genotype than in those with the AA + CA genotypes (Fig. 1). These results suggest that the C allele is associated with the development and activity of GD through the regulation of TRAb production. The frequency of the TT genotype for the rs731236 polymorphism, which was correlated with higher VDR expression, was lower and the frequency of the CC genotype for the rs2228570 polymorphism was higher in HD patients than in GD patients (Table 4). These results suggest that immune regulation by VDR may be suppressed in HD patients in comparison to GD patients. This was supported by the fact that the prevalence of vitamin D deficiency was greater in HD patients than in GD patients [35]. Therefore, genetic differences in the *VDR* gene may be a factor in the development of GD and HD.

For the *GC* polymorphism, the frequency of the Gc1Gc1 genotype, which was correlated with a higher vitamin D concentration, was lower in patients with intractable GD than in those with GD in remission (Table 5). It has been shown previously that vitamin D inhibits the differentiation of Th17 cells and promotes the generation of T_{reg} cells [13]. The possible mechanisms of Th17 suppression may be reduction of IL-6, IL-17A and IL-23 production, and retinoic acid-related orphan nuclear receptor γT ((RORγt) expression [36]. IL-6, IL-17A, transforming growth factor (TGF)-β and RORγt were critical for Th17 differentiation and IL-23 maintains the activity of Th17 cells [37]. Therefore, the suppression of these factors may profoundly affect Th17 cells. In addition, we reported previously that the proportion of Th17 cells in AITD patients, especially in those with intractable GD, was higher than in controls [34]. Therefore, we propose that the suppression of Th17 by vitamin D may inhibit the intractability of GD. Interestingly, for the *CYP2R1* rs10741657 polymorphism, the frequency of the AG genotype (heterozygote) was lower in patients with intractable GD than in those with GD in remission (Table 5). The functional difference between the heterozygosity and homozygosity of this polymorphism is still unclear, which indicates that heterozygosity has some unknown but considerable effects on the pathogenesis of GD. Supporting this possibility, we reported previously that the CA genotype (heterozygote) of the –3279C/A polymorphism in the *forkhead box protein 3 (FoxP3)* gene was associated with the intractability of GD [7].

A limitation of this study may be the small sample numbers. This is because we categorized patients very strictly, and excluded many obscure cases. In this study, however, we could find statistically significant differences between patients' groups despite the moderate numbers of samples, and so we think that such differences would be major.

In conclusion, genetic differences in the *VDR* gene may be involved in the development of AITD and the activity of GD, whereas the genetic differences in the *GC* and *CYP2R1* genes may be involved in the intractability of GD.

Disclosure

The authors have no conflicts of interest to declare.

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